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ZINC-FINGER PROTEIN MCPIP IN CELL DEATH AND DIFFERENTIATION

by

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B.S. Palm Beach Atlantic University, 2003

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Burnett School of Biomedical Science in the College of Medicine at the University of Central Florida Orlando, Florida

Fall Term
2009

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ABSTRACT

Monocyte chemotactic protein-1 (MCP-1) plays a critical role in the development of cardiovascular diseases. How MCP-1 contributes to the development of heart disease is not understood. We present evidence that MCP-1 causes death in cardiac myoblasts, H9c2 by inducing oxidative stress, ER stress and autophagy via a novel Zn-finger protein, MCP-1 induced protein (MCPIP). MCPIP expression caused cell death and knockdown of MCPIP, attenuated MCP-1 induced cell death. Expression of MCPIP resulted in induction of iNOS and production of reactive oxygen (ROS). It caused induction of NADPH oxidase subunit phox47 and its translocation to the cytoplasmic membrane. Oxidative stress led to the induction of ER stress markers HSP40, PDI, GRP78 and IRE1α. ER stress lead to autophagy as indicated by beclin-1 induction, cleavage of LC3 to LCII and autophagolysosome formation. Here, MCPIP-induced processes lead to apoptosis as indicated by caspase 3 activation and TUNEL assay. This cell death involved caspase 2 and caspase 12 as specific inhibitors of these caspases prevented MCPIP-induced cell death. Inhibitors of oxidative stress inhibited ER stress, and cell death. Specific inhibitors of ER stress inhibited autophagy and cell death. Inhibition of autophagy inhibited cell death. Microarray analysis showed that MCPIP expression caused induction of a variety of genes known to be involved in cell death. MCPIP caused activation of JNK and p38 and induction of p53 and PUMA. These results collectively suggest that MCPIP induces ROS/RNS production that causes ER stress which leads to autophagy and apoptosis through caspase 2/12 and IRE1α –JNK/p38-p53-PUMA pathway. These results provide the first molecular insights
into the mechanism by which elevated MCP-1 levels associated with chronic inflammation may contribute to the development of heart failure.

A role for inflammation and MCP-1 in obesity and diabetes has been implicated. Adipogenesis is a key process involved in obesity and associated diseases such as type 2 diabetes. This process involves temporally regulated genes controlled by a set of transcription factors, C/EBPβ, C/EBPδ, C/EBPα, and PPARγ. Currently PPARγ is considered the master regulator of adipogenesis as no known factor can induce adipogenesis without PPARγ. We present evidence that a novel Zn-finger protein, MCPIP, can induce adipogenesis without PPARγ. Classical adipogenesis-inducing medium induces MCP-1 production and MCPIP expression in 3T3-L1 cells before the induction of the C/EBP family of transcription factors and PPARγ. Knockdown of MCPIP prevents their expression and adipogenesis. Treatment of 3T3-L1 cells with MCP-1 or forced expression of MCPIP induces expression of C/EBPβ, C/EBPδ, C/EBPα, PPARγ and adipogenesis without any other inducer. Forced expression of MCPIP induces adipogenesis in PPARγ−/− fibroblasts. Thus, MCPIP is a newly identified master controller that can induce adipogenesis without PPARγ.

Heart failure is a major cause of death in diabetic patients. Hyperglycemia is a major factor associated with diabetes that causes cardiomyocyte apoptosis that leads to diabetic cardiomyopathy. Cardiomyocyte apoptosis is a key event involved in the pathophysiological progression of diabetic cardiomyopathy. We have recently found that in ischemic hearts, MCP-1 can induce the zinc-finger protein, MCP-1 induced protein (MCPIP) that causes cardiomyocyte apoptosis. Although there is evidence that inflammation may play a role in diabetic cardiomyopathy, the underlying mechanisms
are poorly understood. In this study, we show that treatment of H9c2 cardiomyoblasts and Neonatal Rat Ventricular Myocytes (NRVM) with 28mmol/L glucose concentration results in the induction of both transcript and protein levels of MCP-1 and MCPIP. Inhibition of MCP-1 interaction with CCR2 via specific antibody or with the G-coupled receptor inhibitors propagermanium and pertussis toxin attenuated glucose-induced cell death. Knockdown of MCPIP with specific siRNA yielded similar results. Treatment of cells with 28mmol/L glucose resulted in increased ROS production and phox47 activation. Knockdown of MCPIP attenuated these effects. The increased ROS production observed in H9c2 cardiomyoblasts and NRVM’s resulted in increased ER stress proteins GRP78 and PDI. Knockdown of MCPIP attenuated expression of both GRP78 and PDI. Inhibition of ER stress with TUDC and 4’PBA prevented high glucose-induced cell death. Treatment of cells with 28mmol/l glucose resulted in autophagy as determined by an increase in expression of beclin-1 and through increased cleavage of LC3I to LC3II. Knockdown of MCPIP attenuated expression of beclin-1 and prevented cleavage of LC3. Addition of the autophagy inhibitors 3’methyladenine and LY294002 attenuated high glucose-induced H9c2 cardiomyoblast death. We conclude that high glucose-induced H9c2 cardiomyoblast death is mediated via MCP-1 induction of MCPIP that results in ROS that leads to ER stress that causes autophagy and eventual apoptosis.
I dedicate this work to my grandfather, Bill Matheson, who passed away at the beginning of my PhD studies. His advice and wisdom has guided me during my time as a student at the University of Central Florida. I also want to dedicate this work to my wife and parents who have consistently supported me while pursuing my degree.
ACKNOWLEDGMENTS

I would like to thank Dr. Bruce Spiegelman from Harvard University for the PPARγ +/- cell line. The work was supported by the National Institutes of Health (grant HL-69458).
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CHAPTER 1: INTRODUCTION

Ischemic Heart Disease

The Role of Inflammation In Ischemic Heart Disease

Ischemic heart disease (IHD) is the leading cause of heart failure affecting nearly five million Americans. Inflammation plays a critical role in the development of cardiovascular diseases [1-6]. However, the molecular mechanisms are poorly understood. The role of monocyte chemotactic protein-1 (MCP-1) in cardiovascular disease progression is becoming highly recognized [7-9]. There is much evidence implicating the involvement of MCP-1 and its receptor CCR2 in cardiovascular disease development [10-16]. This includes evidence through animal models including CCR2 deficient mice and MCP-1 deficient mice [10-15]. Moreover, cardiac targeted expression of MCP-1 in transgenic mice was found to induce ischemic heart failure with features that mimic human ischemic heart failure [17].

Leukocyte recruitment through MCP-1 binding to its G-protein-coupled receptor CCR2 has been extensively studied [8, 18-21]. Endothelial cells release MCP-1 that tethers to the vascular surface [20, 21]. Monocytes encounter MCP-1 leading to conformational changes in adhesion molecules on both endothelial and monocytic cells. This leads to a tight association and diapedesis of the monocyte into the sub-endothelial layer [8, 18-21]. Signal transduction triggered by MCP-1 binding to its receptor probably results in gene expression changes that most likely play critical roles in the progression of cardiovascular diseases. However, not much is understood about these gene
changes. MCP-1 has been shown to induce production and secretion of the inflammatory molecule IL-1 [22]. MCP-1 treatment of monocytes has been recently reported to induce a novel transcription factor, MCP-1 induced protein (MCPIP), capable of causing cell death [23]. MCPIP contained conserved motifs including a nuclear localization signal (RKKP), a zinc finger (CCCH), and two proline rich regions. MCPIP is suggested to be involved in IHD (Fig. 1).

Figure 1: MCPIP Protein.
MCPIP consists of 2 proline rich regions, a nuclear localization sequence, and a CCCH zinc finger domain.

In MCP-1 transgenic mice, MCPIP transcript levels increase parallel to cardiovascular dysfunction. Furthermore, MCPIP transcript levels increased in human hearts with IHD verses hearts without IHD [23]. However, there is no direct evidence for MCPIP in IHD. This study seeks to find such evidence. MCPIP has been associated with apoptotic cardiomyocytes. If and how MCPIP causes cell death in cardiomyocytes is not known. This study postulates that MCP-1 induces expression of MCPIP in cardiomyocytes causing cell death that may be involved in IHD (Fig. 2).
Cardiomyocyte Cell Death Involved In Ischemic Heart Disease

Cardiomyocyte loss results in decreased contractility of the heart. Understanding and characterizing MCPIP induced cell death can lead to novel therapeutic targets whose intervention can lead to increased cardio function. Three main types of cell death are recognized including apoptosis, autophagy, and necrosis. All have been identified in cardiomyocytes involved in heart failure [24-30].

Figure 2: MCP-1 induces MCPIP that leads to cardiomyocyte death.
MCP-1 signaling interacts with its G protein coupled receptor CCR2 and induces gene expression changes. IL-1 and MCPIP have been shown to be induced by MCP-1 CCR2 interaction.

Apoptosis is characterized by chromatin condensation, DNA fragmentation, membrane blebbing, phosphotydalserine flipping, mitochondrial membrane potential depolarization, and Poly (ADP-ribose) polymerase (PARP) cleavage [31]. Signaling events in apoptosis are classified as either intrinsic or extrinsic (Fig. 3). The extrinsic
pathway utilizes death domain family receptors to activate caspase 8. Caspase 8 can then cleave and activate caspase 3 or 7 which then cleaves PARP leading to the degradation of the cell. The intrinsic pathway involves signaling through the mitochondria. Inhibition of the anti-apoptotic molecule Bcl-2 results in the translocation of Bax and Bak to the mitochondria. This results in the development of the mitochondrial permeable transition pore. Opening of the transition pore releases apoptotic factors including cytochrome C which bind apaf-1 to form the apoptosome. This results in the activation of a caspase cascade[31]. Autophagy has been reported to occur prior to apoptosis [32].

![Figure 3: Intrinsic and Extrinsic Pathways of Apoptosis](image)

Apoptosis can occur through TRAIL binding to its receptor and activating caspase 8 that in turn can activate caspase3/7 directly or via the mitochondria. Intrinsic apoptosis occurs as a result of a stress signal such as p53 causing Bax and Bad translocation to the mitochondria where they signal the opening of mitochondrial transition pores. As a result, cytochrome C is released. Cytochrome C binds to Apaf-1 that binds and activates caspase 9. Caspase 9 can then activate caspase 3/7 thus leading to apoptosis.

Some studies suggest that autophagy initially serves as an effort to protect the cell from undergoing apoptosis, but prolonged exposure drives the cell towards death [32]. Several factors have been identified as initiators of autophagy including the dephosphorylation of Tor and the increased expression of death associated protein kinase (DAPk) and DAPk related protein-1 (DRP-1) [33, 34]. However, the main initiator
of autophagy is beclin-1. Beclin-1, the human homologue to Atg6, interacts with phosphotidylinositol 3-kinase (PI3K) to signal formation of the double membrane lysosomal structure characteristic of autophagy termed the autophagolysosome [32](Fig. 4). This involves numerous autophagy genes such as LC3 I (Atg8) whose cleavage into LC3 II is critical to the development of the autophagolysosome [32]. The autophagolysosome then degrades components of the cell in attempts to eliminate dysfunctional organelles.

Figure 4: Formation of the autophagosome.
Autophagy is characterized by the formation of a double membrane bound structure known as the autophagosome. Beclin-1 interacts with PI3K III to initiate the formation and elongation of the early membrane structure termed the phagophore. Atg7, 4, and 3 cleave LC3 I which then undergoes lipidation and eventually contributes to the completion of the autophagosome structure.

Reactive Oxygen Species And ER stress Involved In Ischemic Heart Disease

In transgenic mice with cardiomyocyte-targeted expression of MCP-1, the development of heart failure was found to be associated with elevated levels of MCPIP and ER stress [35]. ROS production is a known inducer of ER stress and, in turn, ER stress can generate further ROS production directly through the oxidation of disulfide
bonds of PDI by ERO-1 [36] (Fig. 5). This can lead to further amplification of an ER stress response. Prolonged exposure to ER stress has been shown to lead to activation of caspase 12, JNK, and p38 leading to cell death [37-40].

Figure 5: How the ER contributes to ROS
An excessive accumulation of misfolded proteins results in an increase in chaperone proteins such as PDI. PDI reduces disulfide bonds of misfolded proteins. In turn, the disulfide bonds that are now reduced in PDI get oxidized by ERO-1. Through FAD the extra electron is used to convert O2 into H2O2 and thus increases the ROS pool.

IRE1α is a major sensor of unfolded proteins in the ER [41]. Upon activation, it processes the XBP1 mRNA to its mature form. Spliced XBP1 results in the up regulation of ER stress chaperone proteins such as GRP78 [41]. Another function of IRE1α is the activation of JNK and p38 [37, 41]. JNK activation is widely known for its involvement in signaling apoptosis. Furthermore, IRE1α activation of JNK has been reported to initiate autophagy [42]. Because of its ability to signal both apoptosis and autophagy, ER stress has been suggested as a molecular switch between these two
forms of death [43]. This study postulates that MCPIP induces ROS and RNS production resulting in ER stress and ultimately cell death (Fig. 6).

**Figure 6: ROS induces ER stress that can lead to cell death**

ER stress signaling proteins include IRE1, caspase 12 and PERK. All three components are known to induce cell death. IRE1 can induce JNK and p53 while PERK can induce CHOP. CHOP and p53 are known to be involved in apoptosis.

**Hypothesis**

The overall hypothesis of this study is that MCPIP induces cell death in cardiomyocytes. This study seeks to determine if and how MCPIP induces death in cardiomyocytes. It is proposed that MCPIP induces the production of ROS and RNS resulting in ER stress. Furthermore, MCPIP-induced ER stress then results in an IRE1α-JNK/p38-p53-PUMA signaling pathway that results in cell death. The understanding of MCPIP-induced death and the novel genes proposed to be involved in signaling
MCPIP-induced death provides the opportunity for the discovery of novel therapeutic targets to intervene with the progression of IHD.

**Obesity And Type 2 Diabetes**

**Adipogenesis In Obesity And Diabetes**

Approximately 1.1 billion adults are classified as overweight and over 300 million are considered to be obese [44]. Obesity is associated with an increased risk for a set of health complications known as metabolic syndrome and types 2 diabetes. Moreover, the number of individuals with diabetes is expected to reach 300 million by the year 2025. Diabetes is the leading cause of blindness, renal failure and atherosclerotic cardiovascular diseases [45]. Recent years have seen significant increase in the incidence of diabetes mellitus. Excess fat in healthy individuals is stored in adipocyte cells and low amounts of triacylglycerol are maintained in non-adipocytes. In obese patients, the amount of fat that is capable of being stored in the adipocyte tissue can be exceeded resulting in abnormal lipid accumulation in hepatic, pancreatic, and muscular tissues. Abnormal lipid accumulation in these tissue types eventually results in increased dysfunction of those tissues and thus may contribute to the development of type 2 diabetes [46]. There is much evidence showing that adipose tissue has endocrine functions. Adipose tissue is known to secrete hormones, chemokines and cytokines (adipokines) that can regulate metabolic activity in other cell types [47-51]. Understanding the molecular and cellular processes involved in adipogenesis can
provide a clearer understanding of the pathological events that lead to the development of obesity and type 2 diabetes.

An increase in adipose tissue mass is characteristic of obesity [52]. This increase in mass size is contributed to by an increase in the number of fat cells as well as an increase in the size of the fat cells. The process whereby fibroblast-like preadipocytes differentiate into mature adipocytes is known as adipogenesis. Adipogenesis is a process of differentiation systematically controlled by a set of well-characterized transcription factors (Fig. 7).

**Figure 7: Transcription factors involved in adipogenesis.**

C/EBPβ and δ are induced early during adipogenesis followed by C/EBPα and PPARγ. Although both C/EBPα and PPARγ induce genes that result in the mature adipocyte, only PPARγ is considered to be both necessary and sufficient for adipogenesis.

Two transcription factor families have emerged as the key determinants of terminal adipocyte differentiation: the CCAAT/enhancer-binding proteins C/EBPα, -β and -δ, and peroxisome proliferator-activated receptor γ (PPARγ) [53, 54]. As cells undergo differentiation process in response to adipogenic signals, the initial event is the rapid induction of C/EBPβ and -δ expression [55]. A role for C/EBPβ and -δ in the induction of PPARγ2, a key regulator of adipogenesis, has been reported [56, 57]. The importance of C/EBPβ and -δ in adipogenesis was demonstrated by loss-of-function and gain-of-
function genetic studies in mice. Overexpression of either C/EBPβ or -δ in preadipocytes enhanced adipogenesis [54, 55], whereas embryonic fibroblast cells derived from mice lacking either C/EBPβ or -δ had reduced levels of adipogenesis compared with the wild type [14]. Mice lacking both C/EBPβ and -δ showed reduced white adipose tissue mass and reduced lipid staining in brown adipose tissue. Additionally, embryonic fibroblast cells derived from C/EBPβ and δ-double-knockout mice failed to differentiate into mature adipocytes [58]. These studies demonstrated that C/EBPβ and -δ play synergistic roles in adipocyte differentiation and maturation. During the later stage of differentiation, C/EBPα expression rises immediately after PPARγ2 expression. Several studies have demonstrated that PPARγ2 and C/EBPα coregulate each other's expression. Mice with reduced PPARγ expression owing to heterozygous gene-knockout displayed a drastically reduced level of C/EBPα [59]. Likewise, mice with disrupted C/EBPα expression showed a reduced level of PPARγ [60]. The C/EBPα binding site in the PPARγ2 promoter was also shown to bind to C/EBPδ, but not C/EBPβ [57]. The crucial roles of PPARγ and C/EBPα were highlighted by the observation that over expression of either transcription factor in NIH3T3-L1 cells is sufficient to convert these normally nonadipogenic cells from fibroblasts into adipocytes [61, 62]. However, it was unclear whether either transcription factor, completely on its own, could induce adipogenesis. C/EBPα -/- cells failed to undergo adipogenesis, but this defect could be successfully restored by over expression of PPARγ2 [60]. Forced expression of C/EBPα in PPARγ -/- cells, did not cause adipogenesis. These studies demonstrated that PPARγ2 is the key regulator of adipogenesis and C/EBPα induces and maintains PPARγ2 expression [63]. The primary function of C/EBPα could be the
regulation of genes involved in the metabolic actions of insulin, such as glucose transporter 4 (Glut4) [60]. Clearly, PPARγ and C/EBPα are key transcription factors in adipogenesis, acting synergistically to generate fully differentiated, insulin-responsive adipocytes [60, 64]. The research proposed here is likely to discover a third key regulator of adipogenesis.

Inflammation In Obesity And Diabetes

In recent years it has become increasingly clear that obesity involves low-grade systemic inflammatory condition [65]. In addition to the resident macrophages in the adipose tissue, increased accumulation of macrophages in adipose tissue has been found in obese animals and humans. Both adipocytes and macrophages are sources of cytokine production [66]. The bioactive molecules produced by adipocytes (adipokines) include TNFα, leptin, plasminogen activator inhibitor-1, IL-6, resistin and adiponectin. Macrophages secrete IL-1β, TNFα and MCP-1. The macrophages that accumulate in human adipose tissue were reported to represent a unique type that secretes large amounts of proinflammatory cytokines [67]. The proinflammatory cytokines promote cellular insulin resistance in fat, muscle and liver [47-51] (Fig. 8). High levels of MCP-1 production are associated with obesity in animals and humans [68-71]. Recently a role for MCP-1 in obesity-induced insulin resistance was demonstrated [72]. Transgenic mice with adipose tissue-specific expression of MCP-1 exhibited macrophage infiltration into adipose tissue, increased hepatic TG content and insulin resistance. MCP-1 knockout mice fed high fat diet showed drastically reduced macrophage accumulation into adipose tissue and hepatic steatosis when compared to
high fat-fed wild type mice. Furthermore, inhibition of MCP-1 function by acute expression of a dominant-negative mutant of MCP-1 ameliorated insulin resistance in db/db mice and in high fat-fed wild type mice.

![Diagram](image)

**Figure 8: Inflammation in adipocytes leads to insulin resistance**
Adipocyte become enlarged and can no longer efficiently accumulate triglycerides (TG) leading to hypertrophy and MCP-1 secretion. This results in macrophage infiltration that results in an inflammatory state. This then leads to insulin resistance.

These results strongly suggest that increased expression of MCP-1 in adipose tissue causes macrophage infiltration into adipose tissue, insulin resistance and hepatic steatosis associated with obesity in mice. That the role of MCP-1 in obesity-induced diabetes is mediated via its binding to the receptor, CCR2, was demonstrated by results obtained with ccr2−/− mice [73]. CCR2 deficiency attenuated the development of obesity in high fat-fed mice. CCR2 deficiency resulted in reduced macrophage content, increased adiponectin expression, ameliorated hepatic steatosis and insulin resistance. Even in mice with established obesity, short term treatment with a pharmacological antagonist of CCR2 lowered macrophage content of adipose tissue and improved insulin sensitivity. Thus, MCP-1/CCR2 system plays a critical role in obesity-induced diabetes in mice.
Many recent observations suggest that in humans, MCP-1 plays a similar role in obesity-induced diabetes. The increased level of serum MCP-1 levels found in humans correlated with markers of the metabolic syndrome, including obesity, insulin resistance, type 2 diabetes, hypertension, and increased serum TG concentration [74]. Expression of CCR2 on monocytes was reported to be elevated in diabetic patients [71]. Molecular mechanism involved in the role of MCP-1 in obesity-induced diabetes is not understood. MCP-1 could play a critical role in adipogenesis and in promoting growth of new blood vessels to supply blood to the growing adipose tissue.

Hypothesis

We postulate that MCP-1 via interaction with its CCR2 receptor can induce adipogenesis. As a result, MCPIP will be induced. Moreover, MCP-1 induction of MCPIP may be critical event that mediates adipogenesis and thus plays a major role in obesity-induced diabetes.

Diabetic Cardiomyopathy

It is well known that individuals with diabetes have an increased risk for developing heart failure [75-77]. The overall prevalence for heart failure in diabetic individuals is ~12% compared to ~4% in the general population [75]. Moreover, cardiovascular complications are considered to be the leading cause of mortality in diabetic patients [77, 78]. Diabetes is known to alter cardiac structure and function independent of coronary artery disease and hypertension, a condition known as diabetic
cardiomyopathy [77, 79]. Diabetic cardiomyopathy is further defined as a primary
disease process that occurs as the result of metabolic insult that leads to myocardial
abnormalities and eventual heart failure [76].
There are multiple factors that may contribute to the pathophysiological progression of
diabetic cardiomyopathy including autonomic dysfunction, metabolic derangements,
abnormalities in ion homeostasis, alteration in structural proteins, and interstitial fibrosis
[77, 78, 80]. Recent studies suggest that cardiomyocyte loss also plays a key role in
diabetic cardiac damage in both animals and humans [81-85]. Hyperglycemia
contributes to cardiomyocyte death and diabetic cardiomyopathy [86]. Hyperglycemia is
known to cause apoptosis [82-85] that leads to diabetic cardiomyopathy. Attenuation of
hyperglycemia induced cardiomyocyte cell death has been shown to prevent the
progression of cardiac complications associated with diabetes [87]. However, the
molecular mechanisms by which hyperglycemia contributes to development of diabetic
cardiomyopathy have not been fully elucidated.
A role for inflammation in diabetic cardiomyopathy has been implicated [88].
Experimental evidence obtained from CCR2 deficient mice [11, 13], Interestingly,
increased levels of serum MCP-1 have been found in humans to correlate with markers
of the metabolic syndrome, including obesity, insulin resistance, type 2 diabetes,
hypertension, and increased serum TG concentration [74]. Furthermore, endothelial
cells and monocytes exposed to high-glucose levels are known to produce MCP-1 [89].
We recently reported that MCP-1 induces a novel zinc-finger transcription factor, termed
MCPIP [23]. The presence of MCPIP protein and transcripts has been found associated
with apoptotic cardiomyocytes in a murine model of heart failure. Association of MCPIP
with ischemic heart disease was also found in the human disease [23]. How MCP-1 and MCPIP might play a role in the pathophysiological progression of diabetic cardiomyopathy remains unknown.

Hypothesis

This study hypothesizes that hyperglycemia induces cardiomyocyte cell death that contributes to diabetic cardiomyopathy via MCP-1 induction of MCPIP. MCPIP then leads to increased ROS production that results in ER stress that leads to autophagy and eventual apoptosis.
CHAPTER 2: MCP-1 CAUSES CARDIOMYOBLAST DEATH VIA OXIDATIVE STRESS, ER STRESS AND AUTOPHAGY INDUCED VIA A NOVEL Zn-FINGER PROTEIN, MCPIP

Introduction

Inflammation plays a critical role in the development of cardiovascular diseases [1, 2]. The role of leukocyte infiltration and the role of monocyte chemotactic protein-1 (MCP-1) in cardiovascular disease progression are widely recognized [3, 4]. There is overwhelming evidence that MCP-1 is involved in the development of cardiovascular diseases in humans [4, 5]. Many lines of experimental evidence from animal models also provide strong evidence for a critical role for MCP-1 and its receptor CCR2 in the development of cardiovascular diseases. These include results obtained with CCR2 deficient mice [6], MCP-1 deficient mice [7] and transgenic mice with targeted expression of MCP-1 [8]. Death of cardiomyocytes is known to be involved in heart failure. We recently reported that treatment of monocytes with MCP-1 induces a novel Zn-finger protein, MCP-1 induced protein (MCPIP) that can induce cell death [9]. The presence of MCPIP transcripts and protein was associated with the apoptotic cardiomyocytes in a murine model of heart failure with cardiomyocyte-targeted expression of MCP-1. Association of MCPIP with ischemic heart disease was also found in the human disease [9]. Although such indirect indications suggest a role for MCPIP in causing cell death associated with heart failure, how MCPIP might cause cell death is not known.
In transgenic mice with cardiomyocyte-targeted expression of MCP-1, the development of heart failure was found to be associated with elevated levels of MCPIP [9] and histochemically detectable reactive oxygen species (ROS) and reactive nitrogen species (RNS) production [10]. Oxidative stress is a known inducer of ER stress [11, 12] and the MCP-1 transgenic mice showed ER stress prior to developing heart failure [13]. ER stress is known to cause autophagy [14, 15] that can lead to cell death [16, 17]. On the basis of such observations on MCP-transgenic mice and the literature, we postulate that MCP-1 would cause cell death via MCPIP induction, ROS production, ER stress and autophagy. Since it is more convenient to test the validity of such molecular mechanisms in cell cultures, we tested this hypothesis in cardiomyoblast cell line, H9c2 cells. We show that MCP-1, via MCPIP expression, induces ROS/RNS production that causes ER stress, that leads to autophagy and apoptosis in H9c2 cells. We present evidence that MCPIP-induced ER stress leads to the death of H9c2 cells through caspase 2/12 and IRE1α – JNK/p38 – p53 – PUMA pathway. These results provide the first molecular insight into the probable mechanism by which elevated levels of MCP-1 associated with chronic inflammation may cause cardiomyocyte death and thus contribute to the development of heart failure.

Material and Methods

Adenoviral Mediated Expression of MCPIP-GFP and GFP

The plasmid containing the MCPIP-GFP cDNA (pMCPIPGFP) has been previously described [9]. The adenovirus shuttle vector pACCMV-IRESGFP (from Dr. Robert Gerard) containing the adenovirus serotype 5 genome and the cosmid CS360
(from Dr. Gary Nabel) were used. The pMCPIPGFP cDNA was inserted at the EcoRI and BamHI sites of pACCMV-IRESGFP to yield the plasmid pCMVMCPIPGFP. Cre-lox recombination was performed in vitro and the resulting plasmid (adenoviral MCPIP plasmid) was kept for transfection of HEK293 cells which contained the compliment adenovirus genome sequence needed to package the virus. HEK 293 cells were cotransfected the adenoviral MCPIP plasmid using a calcium phosphate transfection system (Life Technologies, Inc.). After 5 h, the cells were washed three times with medium. The medium was replaced every 3 days. After 21 days the cells were harvested for cell lysate preparation. The cells were frozen and thawed 3 times and centrifuged at 5,000g for 10 min, and the crude lysate, containing the recombinant adenovirus (AdMCPIPGFP), was used to infect confluent HEK 293 cells.

Amplification was performed in HEK293 cells, and purification was done using BD Adeno-X virus purification kit (Clontech). Tittering was done through transfection of HeLa Cells with a serial dilution of the adenoviral stock and assessing the expression of GFP. A multiplicity of infection of 20, gave optimal levels of expression of the fluorescent protein in H9c2 cells.

Cell Culture, Adenoviral Infection, and Measurement of Cell Death

H9c2 rat cardiomyoblast (ATCC) were grown in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 2% FBS, 1% penicillin and 1% streptomycin, and infected with either MCPIP-GFP or GFP adenovirus at an MOI of 20. After 6 hours of infection, cells were grown in DMEM supplemented with 10% FBS, 1% penicillin and 1% streptomycin. Cells were treated with appropriate inhibitors 3 hours prior to
adenovirus transfection (Table 1). Cell viability and death were measured by (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, trypan blue and TUNEL assays using standard procedures.

**Table 1: Inhibitors and Concentrations**

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Concentration</th>
</tr>
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<tbody>
<tr>
<td>3’-methyladenine (3’MA)</td>
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</tr>
<tr>
<td>4-phenyl butyric acid (PBA)</td>
<td>50µmol/L and 100µM</td>
</tr>
<tr>
<td>Tauroursodeoxycholate (TUDC)</td>
<td>50µmol/L</td>
</tr>
<tr>
<td>Z-ATD-FMK</td>
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</tr>
<tr>
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<tr>
<td>1400W</td>
<td>50µmol/L</td>
</tr>
<tr>
<td>Tiron</td>
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</tr>
<tr>
<td>JNK III inhibitor</td>
<td>100µmol/L</td>
</tr>
<tr>
<td>SB203580</td>
<td>10µmol/L</td>
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</tbody>
</table>

**Cell Death Microarray Analysis**

The nonradioactive rat cell death oligomicroarray (Super Array; Biosciences Corp.) was used. H9c2 cells were infected with adenovirus expressing MCPIP–GFP or GFP alone for 72 hours before the isolation of RNA. The expression profiling was done according to manufacturer's instructions.
ROS Measurements

H9C2 cells were infected with MCPIP-GFP or GFP adenovirus for 24 hr and after different periods, ROS was measured with 1 µmol/L Dihydrorhodamine 123 (DHR123) for 30 min. at 37°C and 5% CO₂ and were evaluated with a fluorometric plate reader (Excitation 550 nm and Emission 590 nm).

RT-PCR

Total RNA was isolated with the RNAeasy kit (Quiagen) from H9c2 cells expressing MCPIP-GFP or GFP alone, and first-strand cDNA was synthesized using 1 µg total RNA (DNase-treated) using I Script cDNA synthesis kit (Bio-Rad); GADPH served as internal control. Primers designed for RT-PCR are listed Table 2.

Table 2: RT-PCR Primers

<table>
<thead>
<tr>
<th>Forward 5’ – 3’</th>
<th>Reverse 5’ – 3’</th>
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</tr>
<tr>
<td>HSP40</td>
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</tr>
<tr>
<td>PDI</td>
<td>CAGAATGGAAACCACGCAAAACCCA GGCCACATCCACCCTCAAAACAA</td>
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</table>
**In Vivo Measurement of Caspase 3 Activity**

H9c2 cells \((5 \times 10^5)\) were plated on 6 well plates for 24hr and were infected with MCPIP-GFP or GFP expressing adenovirus as indicated above. After 72 hours, cells were treated with 1µL Red-DEVD-FMK and incubated for 1hr. at 37\(^\circ\)C at 5% CO\(_2\). Cells were washed twice, and were then assessed on flurometric plate reader (Ex. 540nm and Em. 570nm).

**Immunoblot Analysis**

H9c2 cells were treated with cell lysis buffer (20% glycerol 0.1% TritonX 8% 0.5M EDTA and 1% 1M DTT) and protein samples were collected and subjected to immunoblot using the polyclonal antibodies listed in Table 3. Immunoblots were quantified as a ratio over GAPDH.

**Table 3: Immunoblot Primary Antibodies**

<table>
<thead>
<tr>
<th>Target</th>
<th>Source</th>
<th>Concentration</th>
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<tbody>
<tr>
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</tr>
<tr>
<td>Caspase 2</td>
<td>Santa Cruz</td>
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</tr>
<tr>
<td>ERO1</td>
<td>Novus</td>
<td>1:2000</td>
</tr>
<tr>
<td>iNOS</td>
<td>Upstate</td>
<td>1:2000</td>
</tr>
<tr>
<td>IRE1</td>
<td>Santa Cruz</td>
<td>1:1000</td>
</tr>
<tr>
<td>P-c-Jun</td>
<td>Upstate</td>
<td>1:1000</td>
</tr>
<tr>
<td>c-Jun</td>
<td>Upstate</td>
<td>1:2000</td>
</tr>
<tr>
<td>LC3</td>
<td>MBL International</td>
<td>1:2000</td>
</tr>
<tr>
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</tr>
<tr>
<td>p53 (F1393)</td>
<td>Santa Cruz</td>
<td>1:2000</td>
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<tr>
<td>PUMA</td>
<td>Cell Signaling</td>
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siRNA Treatment

H9c2 cells were transfected with 100nmol/L of a chemically synthesized siRNA targeted for the appropriate gene (Ambion) or with 100nmol/L non-specific siRNA (Ambion) using Dharmafect for 24hr prior to infection with MCPIP-GFP or GFP expressing adenovirus.

Statistical Analysis

The experimental data was analyzed by using SPSS statistical software (SPSS Inc.) under Windows XP. All values are presented as mean ±SEM. Results were compared between groups by ANOVA analysis followed by t tests. Differences were considered significant at a P value of <0.05.

Results

MCP-1 Treatment of H9c2 Cells Caused Cell Death Via MCPIP Induction

Since elevated MCP-1 expression is associated with ischemic cardiomyopathy that is known to involve death of cardiomyocytes, we tested wether MCP-1 treatment of H9c2 cells can cause cell death. MCP-1 treatment of H9c2 cells caused cell death (Fig. 9A). To determine whether the death caused by MCP-1 treatment is mediated via MCPIP, cells were treated with MCP-1 and siRNA specific for MCPIP. We found that MCP-induced cell death in H9c2 was attenuated by siRNA specific for MCPIP (Figure 1A) whereas nonspecific siRNA showed no effect. Adenovirus mediated expression of MCPIP-GFP or GFP was observed by the appearance of fluorescence after infection of the H9c2 cells. Expression level of the adenovirus mediated MCPIP-GFP was maximal
at 1 day after infection and subsequently decreased (Figure 9B). Immunoblot analyses revealed the production of the expected fusion protein (Fig. 9C). Adenoviral expression of MCPIP-GFP in H9c2 decreased viability as measured by the MTT assay (Fig. 9D) and caused cell death as measured by trypan blue (Fig. 9E). Since MCPIP-expressing cardiomyocytes were found to undergo apoptosis in transgenic mice with cardiomyocyte-targeted expression of MCP-1 [9], we tested whether MCPIP expression induced apoptosis in H9c2 cells.

**Figure 9: MCP-1 induced H9c2 cell death that was mediated via MCPIP**

(A), Cells were treated with 100nmol/L of siRNA specific for MCPIP, or 100nmol/L non specific siRNA 12 hours prior to treatment with 10ng/mL MCP-1. Cells were evaluated 24 hours post MCP-1 treatment for cell death using trypan blue (p<0.01). (B), Cells were infected with 20MOI of MCPIP-GFP or GFP alone containing adenovirus. Fluorescence was monitored using microscopy and the percentage of cells positive for expression was recorded 1-5 days. (C), H9c2 cells were infected with MCPIP-GFP or GFP alone, and 50µg of cell lyses was subjected to immunoblot with antibody against MCPIP. H9c2 cells expressing MCPIP-GFP were examined for cell viability using MTT assay (D) or for cell death using trypan blue (E) (p<0.02) and TMR TUNEL (F).
Expression of MCPIP-GFP in H9c2 resulted in TUNEL positivity from 1 to 3 days after infection, with maximal positivity occurring at 3 days. (Fig.9F). These results indicate that MCP-1 caused cell death via MCPIP.

MCPIP induces the production of ROS

Since ROS is a major signaling component often associated with cell death, we tested whether MCPIP expression caused ROS production. Immunoblot analysis showed that MCPIP-expressing cells had increased iNOS protein levels beginning on day 1. (Fig.10A-B). An increase in NADPH oxidase phox47 subunit protein level was observed (Fig. 10C). Activation of NADPH oxidase phox47 subunit occurred 1 day after MCPIP-adenoviral transfection as indicated by translocation of phox47 from cytoplasm into the plasma membrane (Fig. 10D). MCPIP-induced ROS production was demonstrated with the fluorescent dye, DHR123 (Fig. 10E). Inhibition of iNOS and NADPH oxidase was able to attenuate ROS production (Figure 10E-G). These results indicate that MCPIP expression induces iNOS and NADPH oxidase that lead to ROS production.

MCPIP-induced ROS causes an ER stress response

Since MCP-1 has been reported to induce an ER stress response in the heart tissue of mice with cardiomyocyte-targetted expression of MCP-1 [13], we tested whether MCP-1 treatment of H9c2 cells causes ER stress. MCP-1 treatment of H9c2 cells resulted in increased expression of the ER stress markers Hsp40, PDI, and GRP78 at the transcript level (Fig. 11A).
Figure 10: MCPIP induced ROS/RNS production.

(A). MCPIP-GFP or GFP alone were expressed in H9c2 cell line using adenovirus mediated gene delivery at 20 MOI. 20µg of cell lysate was subjected to immunoblot using antibody against iNOS. (B). Quantitation of immunoblot normalized against GAPDH (p<0.05) (C), 40µg of protein was evaluated via immunoblot with antibody specific for Phox47. Quantitation of immunoblot normalized against GADPH (p<0.02). (D), Membrane fractions were isolated from total cell lysate, and 20µg of protein was evaluated via immunoblot for phox47 or the membrane fraction control cox4. Quantitation of the immunoblot normalized to cox4. (E-G) , Cells were treated with or without iNOS inhibitor 1400W, NADPH oxidase inhibitor Tiron, and 100nM CeO2 nanoparticles 1-3 hours prior to infection. Total ROS was detected by treating the MCPIP-GFP or GFP expressing cells 48hours post infection with 10µmol/L DHR123 for 30min. Cells were then examined fluorometrically (excitation 550nm; emission 590nm/p<0.01).
Figure 11: MCPIP induced an ER stress response.
(A), H9c2 cells were treated with siRNA specific for MCPIP or nonspecific siRNA (NS) prior to treatment with or without 10ng/mL MCP-1. After 24hrs, cells were evaluated for the ER stress proteins HSP40, PDI, and GRP78 using real-time RT-PCR (p<0.05). (B,C), Cell lysate from H9c2 cells with adenovirus-mediated expression of MCPIP-GFP or GFP alone (20MOI) was evaluated using immunoblot to detect HSP40, PDI, and GRP78.

This induction was attenuated by knockdown of MCPIP expression with siRNA indicating that MCP-1 induced ER stress via MCPIP. Moreover, H9c2 cardiomyoblasts expressing MCPIP-GFP showed marked induction in protein levels of the ER chaperones HSP40, PDI and GRP78 as compared to GFP controls (Fig. 11B and C). These results indicate that MCP-1 causes an ER response via induction of MCPIP. Since IRE1α is known to be a major signaling component involved in ER stress-
mediated events [18, 19], we measured this protein level. Cells expressing MCPIP-GFP showed significantly elevated levels of this protein (Fig. 12A). Moreover, the ER-stress associated caspase 2 [20] was activated in cells expressing MCPIP (Fig. 12B and C). Caspase 2-specific inhibitor VDVAD-FMK inhibited MCPIP-induced activation of caspase 2 in H9c2 (Fig. 12B).

![Image of Western Blot Analysis](image)

**Figure 12: MCPIP induced the ER stress protein IRE1α and induces cleavage of caspase 2.**

(A), MCPIP-GFP or GFP alone were expressed in H9c2 cell line using adenovirus mediated gene delivery at 20 MOI. Cell lysate (20µg protein) was subjected to immunoblot analysis using antibody against IRE1α. (B,C), H9c2 cells were treated with or without 10µmol/L of the caspase-2 inhibitor Z-VDVAD-FMK (Kamiya Biomedical) 1 hour prior to infection with GFP or MCPIP-GFP expressing adenovirus. Media was changed daily with or without Z-VDVAD-FMK for a period of 3 days. After 3 days cells were collected and evaluated for caspase 2 activity via immunoblot analysis of caspase 2 cleaved product. The immunoblot results were quantified against GAPDH.
Figure 13: Inhibition of ROS production attenuated MCPIP-induced ER stress and inhibition of ER stress attenuated ROS production.

(A, B, C), H9c2 cells were treated with or without 1400W or tiron1hr. prior to infection with adenovirus expressing MCPIP-GFP or GFP alone and real-time RT-PCR was used to assess levels of the ER stress proteins HSP40, PDI, and GRP78 2days post infection (B. * = p<0.03) (C. * = p<0.04). (D), Cells were also treated with TUDC 1hr. prior to adenovirus infection and were evaluated for ROS production using 10µmol/L DHR 123 incubated for 30 min. and then examined fluorometrically (excitation 550nm; emission 590nm/p<0.01)

We next tested whether MCPIP-induced ROS production is required for the induction of ER stress in H9c2 cells. We found that inhibition of iNOS with 1400W and inhibition of ROS production with tiron resulted in a significant decrease in the expression levels of Hsp40, PDI, and GRP78 in MCPIP-expressing cells (Fig. 13A and B). Furthermore, inhibition of MCPIP induced-ROS/RNS production with CeO₂ nanoparticles also resulted in a decrease in expression levels of Hsp40, PDI and
GRP78 (Fig. 13C). Interestingly, inhibition of ER stress with the ER stress specific inhibitor tauroursodeoxycholate (TUDC) resulted in reduced ROS production as well (Fig. 13D). Moreover, the ER stress protein ERO1 increased in MCPIP-expressing cells (Fig. 14A). Furthermore, treatment of these cells with siRNA for ERO1, a critical player in ER stress, resulted in decreased ROS production (Fig. 14B).

![Image](image_url)

**Figure 14: MCPIP induced the ER protein ERO1.**
(A), H9c2 cells were infected with adenovirus expressing MCPIP-GFP or GFP alone. Cell lysate prepared 48hrs after infection (20μg of protein) was evaluated using immunoblot to detect ERO1. (B) H9c2 cells were treated with siRNA specific for ERO1 prior to adenovirus infection. ROS was evaluated fluorometrically using DHR123 (excitation 550nm; emission 590nm) (p<0.01).

**Expression of MCPIP in H9c2 Induces Autophagy**

Since ER stress is known to lead to autophagy and autophagy has been reported to occur in cardiomyocytes involved in cardiovascular disease [17, 21], we tested whether MCPIP-induced death in H9c2 cells involves increased formation of autophagolysosomes characteristic of autophagy. Cells with MCPIP expression showed higher amounts of autophagosomes than did GFP control (Fig. 15A). Knockdown of
beclin-1 with siRNA attenuated the accumulation of MCPIP induced-autophagolysomes (Fig. 15A). We found that beclin-1, an autophagy marker, significantly increased 2 days after adenovirus infection (Fig. 15B and 15C). LC3 cleavage to LC3 II was also induced by MCPIP (Fig.15D and 15E). To test whether the autophagy induced by MCPIP is mediated via ER stress, we tested whether ER stress inhibitor TUDC, would inhibit autophagy. Induction of beclin1 by MCPIP was inhibited by TUDC and by knockdown of IRE1 with siRNA (Fig. 15F).

Expression of MCPIP in H9c2 induced apoptotic cell death via ROS, ER stress, and autophagy

We tested whether cell death resulting from MCPIP expression is mediated via ROS induced ER stress that leads to autophagy. Inhibition of oxidative stress with either the iNOS specific inhibitor 1400W or CeO₂ nanoparticles resulted in the attenuation of MCPIP-induced cell death (Fig. 16A). Specific inhibitors of ER stress, 4-phenylbutyric acid (4-PBA) and TUDC [22] (Fig. 16B and C) and knockdown of IRE1 with siRNA (Fig. 17C and D) attenuated MCPIP-induced cell death. We tested whether a specific inhibitor of ER stress associated caspases 2 and 12 would inhibit MCPIP-induced cell death. In fact, VDVAD-FMK and Z-ATD-FMK attenuated MCPIP-induced cell death in H9c2 cells (Fig. 16D and E). Moreover, 3’methyladenine (3’MA) (Fig. 16F), that is known to inhibit autophagy, and knockdown of beclin-1 (Fig. 17C and D) also resulted in the attenuation of MCPIP-induced cell death. In support of this conclusion, treatment of MCPIP-GFP expressing H9c2 with 1400w, TUDC or 3’MA showed marked inhibition of apoptosis as detected by TMR TUNEL (Fig. 17A and 17B). Furthermore, treatment of MCPIP-GFP expressing H9c2 with 1400W, TUDC or 3’MA resulted in decreased
caspase 3 activity (Fig. 17B). We conclude that MCPIP induces ROS that results in ER stress that leads to autophagy and eventual apoptosis.

Figure 15: MCPIP induced autophagy via ER stress.
H9c2 cells were infected with adenovirus expressing GFP or MCPIP-GFP and were evaluated 2 days later. (A) At 48 hrs after infection cells were stained with monodansylcadaverine (MDC) to detect autophagolysosome formation. Cells were then assessed microscopically. (B). Cell lysates (50µg protein) were subjected to immunoblot with antibody against beclin-1. (C), Quantitation of immunoblot data in B, normalized against GAPDH (p<0.01). (D), 20µg of protein was evaluated via immunoblot to detect cleavage of LC3 I into LC3 II. (E), Quantitation of immunoblot data in D normalized against GAPDH (p<0.02). (F), Cells were treated with TUDC prior to adenovirus infection. 50µg of cell lysates were subjected to immunoblot with antibody against beclin-1.
Figure 16: MCPIP induced cell death occurred via ROS induction of ER stress that lead to autophagy.

H9c2 cells were infected with adenovirus expressing GFP or MCPIP-GFP. Cells were treated with or without iNOS inhibitor 1400W or CeO2 nanoparticles 1-3 hours prior to infection. (A), Cell death was evaluated using trypan blue 72hrs. after infection (p<0.02) with or without treatment with 1400W or CeO nanoparticles. Cells were treated with 4PBA (B) p<0.01), TUDC (C) p<0.02), VDVAD-FMK (D) p<0.02) or Z-ATD-FMK (E) p<0.01) 1-3hr. prior to infection with MCPIP-GFP or GFP-expressing adenovirus (20MOI). Samples were examined for cell death 3 days post infection using trypan blue. (F), Cells were treated with 50µM 3’MA 1 hr. prior to adenovirus mediated expression of MCPIP-GFP or GFP. Cells were evaluated 1-3 days post infection for cell death using trypan blue (p<0.03).
Figure 17: MCPIP induced cell death involved caspase 3 activation and apoptosis.

(A,B). Cells were treated with or without 1400W, TUDC or 3′MA 1-3hr. prior to infection at 20MOI with MCPIP-GFP or GFP expressing adenovirus, and were evaluated for apoptosis using TMR TUNEL (B, left, p<0.04). (B, right), After 72hrs, caspase 3 was monitored in cells using Red-DEVD-fmk (p<0.02).

(C, D). Cells were treated with or without siRNA specific for IRE1 or Beclin1 prior to infection at 20MOI with MCPIP-GFP or GFP expressing adenovirus, and after 72 hrs were evaluated for apoptosis using TMR TUNEL (D, left, p<0.04). (D, right), After 72 hrs, caspase 3 was monitored in cells using Red-DEVD-FMK (p<0.02).
MCPIP expression causes activation of JNK and p38 and induction of p53 and PUMA

Microarray analysis done with RNA isolated 3 days after adenoviral infection revealed that MCPIP induced many of the genes that are known to be important in signaling cell death (Table 4). Beclin-1 and p53 showed the highest levels of fold increase in expression. Caspase 3 increased 2.3 fold and caspase 9 increased 5.2 fold. The ER associated caspases 4 and 12 also increased.

Table 4: MCPIP induced expression of genes involved in cell death.

<table>
<thead>
<tr>
<th>Gene Family</th>
<th>Gene</th>
<th>Fold Expression</th>
<th>Description</th>
</tr>
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<tr>
<td>BCL2</td>
<td>BAD</td>
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<td>BCL-2 Associated Domain</td>
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<tr>
<td></td>
<td>BAG1</td>
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Because IRE1α signals JNK activation [18, 19], we examined its role in MCPIP-induced death. We also examined p38 that is known to be induced by ER stress [24]. Inhibition of both JNK and p38 attenuated MCPIP-induced cell death (Fig. 18A). Furthermore, levels of phosphorylated-c-jun increased in cells expressing MCPIP-GFP as compared to GFP controls, while overall levels of c-jun remained unchanged (Fig. 18B). Moreover, the downstream proteins p53 and PUMA, regulated by JNK and p38 activation [25], increased in MCPIP-expressing cells compared to GFP controls (supplemental Fig. 18C). Thus, MCPIP-induced ER stress probably signals cell death through JNK/p38 activation and consequent induction of p53 and PUMA (Fig. 19).

Figure 18: MCPIP induces death via the cell death signaling proteins JNK/p38, p53 and PUMA.

(A). Cells expressing MCPIP-GFP or GFP were treated with or without 100µmol/L JNKII inhibitor or the p38 inhibitor 10µmol/L SB203580 3 hrs. prior to adenoviral infection and after 72hrs were evaluated for cell death using trypan blue (p<0.01). (B), Immunoblot with Phosphorylation specific antibodies shows phosphorylated c-jun, the direct target of active JNK. Levels were compared to the unphosphorylated form of c-jun. (C), Immunoblot analysis shows upregulation of JNK’s downstream targets p53 and PUMA.
**Figure 19: Conclusion**
MCPIP induces ROS that causes ER stress that leads to autophagy and eventual cell death.
Discussion

If and how MCP-1 plays a role in the death of cardiomyocytes that is known to be involved in heart failure is unknown. In addition, the signaling events resulting from MCP-1/CCR2 interaction might lead to gene expression changes that may play a critical role in the development of cardiovascular diseases. The nature and the role of such gene expression changes in the pathophysiological changes associated with heart disease are poorly understood. We recently reported that MCP-1 induces MCPIP, a novel Zn-finger protein that has transcription factor-like activity [9]. In mice with cardiac targeted expression of MCP-1, MCPIP expression was elevated and was associated with cardiomyocyte apoptosis and heart failure [9]. If and how MCPIP might cause cardiomyocyte death is not known. We postulate that MCPIP induces oxidative and nitrosative stress that would cause ER stress which would lead to autophagy and cell death, and present experimental evidence to support this hypothesis.

Recent reports demonstrated involvement of ROS and RNS in myocardial infarction, cardiac hypertrophy, cardiomyopathy, and heart failure in animals and human patients [26-28]. Moreover, it was recently found that ROS and RNS production in ischemia/reperfusion was attenuated in CCR2/-/- mice. Here we show that MCPIP induces ROS production in the cardiomyoblast cell line H9c2 via induction and activation of phox47. This idea was further supported by the finding that inhibition of iNOS with 1400w and NADPH oxidase with tiron attenuated ROS production and cell death. ROS and RNS production is known to cause ER stress [11, 12, 29, 30]. It was recently reported that hypoxia in cultured myocytes and MI in intact animals caused ER
stress in cardiomyocytes [30]. ER stress results from the accumulation of misfolded proteins which lead to the induction of the unfolded protein response (UPR) [31]. The present results demonstrate that MCP-1 treatment of H9c2 cells results in increased expression of the ER stress proteins ERO1, HSP40, PDI, and GRP78 at both transcript and protein levels. That induction of ER stress by MCP-1 was mediated via MCPIP was shown by the finding that knockdown of MCPIP inhibited MCP-1 induced ER stress. Furthermore, expression of MCPIP in H9c2 cells induced ER stress chaperones. We showed that MCPIP-induced ER stress was mediated via ROS and RNS production. Treatment with CeO₂ nanoparticles attenuated oxidative stress, ER stress, and cell death. Furthermore, use of the recently reported specific ER stress inhibitors [22], also resulted in decreased ROS production in cells expressing MCPIP. This result can be explained by the induction of ERO1 which is known to generate ROS production by the reduction of the disulfide bonds in PDI [19, 32]. In fact, knockdown of ERO1 with siRNA resulted in a decrease of ROS production. Thus MCPIP induces ROS production that causes ER stress that leads to further amplification of ROS production through ERO1. In addition, inhibition of ER stress by specific inhibition or by knockdown of IRE1 resulted in the attenuation of MCPIP-induced cell death. Thus our results indicate that MCPIP induces ROS production that causes ER stress that leads to cell death.

Even though the critical role of MCP-1 in the development of cardiovascular diseases is well recognized, there are observations that suggest a protective role for MCP-1. Our finding, that MCP-1 induces the expression of ER stress chaperones via MCPIP, can reconcile these seemingly contradictory results. In isolated neonatal mouse cardiac myocytes, treatment with MCP-1 protected the cells from death caused by
subsequent 18 hr hypoxia [33]. This protection could arise from MCP-1 induced ER stress chaperones. Many reports have demonstrated that expression of ER stress chaperones have cardioprotective effects. For example, protection by ischemic preconditioning by the myocardial expression of GRP78 and protection from ischemia reperfusion injury by cardiac expression of ER stress chaperone have been demonstrated [34, 35]. It has been demonstrated that expression of ER stress chaperone GRP78 prior to ischemic injury protects cells [36], whereas sustained and prolonged stress would result in the breakdown of this protection. Cardiac-targeted expression of MCP-1 in transgenic mice shows cardioprotective effects against myocardial infarction and ischemia/reperfusion damages in young animals [37-39]. In such animals ER stress chaperones have been shown to be elevated [13] and this elevated chaperone levels probably account for the observed protection. However, as the animals age the sustained myocardial expression of MCP-1 would lead to prolonged oxidative and nitrosative stress under which protection would break down with a decrease in ER stress chaperones as was found [13]. This loss of protection would cause autophagy and cell death leading to the development of heart failure and death of the animal at about 6 months of age.

ER stress is known to cause cell death through multiple signaling pathways [19, 24, 40]. Caspase 2 activation has been reported to be involved in oxidative stress-induced apoptosis [41]. Caspase 2 and caspase 12 are ER-related caspases that have been shown to be activated upon prolonged ER stress resulting in cell death [20, 23]. Caspase 2 has been recently shown to cleave and activate Bid, a BH3 pro-apoptotic protein that activates the mitochondrial apoptotic proteins Bax and Bak [20]. Our
microarray analysis showed that MCPIP expression induced production of caspase 12 and immunoblot analysis showed that caspase 2 was cleaved into its active form in MCPIP-expressing H9c2 cells. In addition, specific inhibition of caspase 12 or caspase 2 resulted in attenuation of MCPIP-induced cell death. Expression of MCPIP in cardiomyoblasts resulted in the induction of IRE1 protein which is a well known signaling molecule involved in ER stress induced cell death [18, 19]. IRE1 can induce autophagy and apoptosis [18, 40]. ER stress is known to cause autophagy [14, 15]. Autophagy is known to be involved in the death of cardiomyocytes that leads to heart failure [16, 17, 21]. Beclin-1 is the key protein involved in autophagy and can interact with the antiapoptotic protein Bcl2 and its binding state may play a key part in coordinating the cellular decision to undergo autophagy [42]. Activation of autophagy through beclin-1 results in the accumulation of autophagosomes, an event that requires the cleavage of the autophagy protein LC3 [40]. Our results showed that expression of MCPIP in cardiomyoblasts resulted in increased expression of beclin-1 protein levels and cleavage of LC3. The finding that inhibition of autophagy by 3'-MA and knockdown of beclin-1 with siRNA resulted in attenuation of MCPIP-induced death strongly implicates autophagy in cell death. This conclusion was supported by our results showing that inhibition of MCPIP-induced of ER stress with TUDC and by knockdown of IRE1 with siRNA resulted in decreased expression levels of beclin-1 and cell death.

The precise role of autophagy in cell death is unclear. Some reports suggest that autophagy attempts to protect the cell from death by degrading misfolded proteins [43, 44]. Autophagy is also known to cause cell death under the influence of developmental or stress signals [45]. It is known that signaling components of apoptosis can, in fact,
induce autophagy [46]. Apoptosis has been associated with heart failure [47]. Our results show that MCPIP expression in cardiomyoblast resulted in apoptosis as detected by TUNEL and caspase 3 cleavage. Moreover, many of the genes that were induced by MCPIP, as revealed by our microarray analysis, can be either apoptotic or related to autophagy. Inhibition of ER stress with TUDC resulted in decreased caspase 3 activity, demonstrating that MCPIP-induced ER stress does cause apoptosis. Signaling components previously shown to be induced by the ER stress protein, IRE1, were also found to be activated or induced in MCPIP-expressing cells. These included phosphorylation of c-JUN and increased expression levels of p53 and PUMA. Moreover, a role for caspase 2 in p53-mediated apoptosis has been recently reported [48]. Thus, molecular players involved in cardioblast death induced by MCP-1 can be depicted as shown in scheme 1.

It is likely that the processes elucidated here in H9c2 cells occur in vivo. In the transgenic mice with cardiomyocyte-targetted expression of MCP-1, the failing heart tissue showed ROS production, as indicated by staining of heart tissue with chloromethyl dichlorofluorescein diacetate, and RNS production, as indicated by staining for nitrotyrosine [10]. These hearts showed elevated expression of ER stress chaperones prior to the development of significant left ventricular dysfunction [13]. Inhibition of oxidative stress by administration of CeO2 nanoparticles to the transgenic mice inhibited ER stress development, cardiomyocyte apoptosis, and development of heart disease [8]. Thus, the molecular mechanisms underlying the MCP-1 induced death in H9c2 cells elucidated here are probably involved in intact animals and form the molecular basis of how the elevated levels of MCP-1 associated with chronic
inflammation contributes to the development of ischemic heart failure by causing the death of cardiomyocytes.
CHAPTER 3: MCPIP, A NOVEL MASTER CONTROLLER THAT CAN INDUCE ADIPOGENESIS WITHOUT PPARγ

Introduction

The incidence of obesity and associated diseases is increasing dramatically throughout the world. The global incidence of type 2 diabetes is projected to double to 350 million cases by the year 2030 [1]. Because adipose tissue serves as a crucial integrator of glucose homeostasis, understanding of adipocyte biology is crucial for understanding the pathophysiological basis of obesity and related metabolic diseases [2, 3]. Adipogenesis and angiogenesis, which supply blood to the growing adipose tissue, occur in the growing adipose tissue when the caloric intake significantly exceeds caloric expenditure. Adipogenesis involves a temporally regulated set of gene expression controlled by a set of transcription factors [3, 4]. C/EBPβ and C/EBPδ are induced early, followed by C/EBPα and PPARγ. It is widely accepted that PPARγ is the master regulator of adipogenesis [2, 4]. This conclusion is based mainly on the finding that forced expression of PPARγ in C/EBPα−/− fibroblasts induces adipogenesis [5], whereas forced expression of C/EBPα in PPARγ−/− fibroblasts does not induce adipogenesis [6]. Thus, the current concept is that PPARγ is both necessary and sufficient for adipogenesis. No other factor is known that can induce adipogenesis in the absence of PPARγ [7]. Here we report that a newly discovered zinc finger protein, MCP-1-induced protein (MCPIP) [8], induces adipogenesis in the absence of PPARγ. In an engineered tissue model, MCP-1 treatment was reported to enhance adipose tissue volume [9]. In
3T3-L1 cells undergoing adipogenesis in the commonly used adipogenesis-inducing medium, containing dexamethasone, 3-isobutyl-1-methylxanthine and insulin (DMI), MCP-1 is produced and MCPIP is induced before the induction of the C/EBP family of transcription factors and PPARγ. Knockdown of MCPIP with siRNA inhibits induction of these genes and adipogenesis. Treatment of 3T3-L1 cells with MCP-1 or forced expression of MCPIP induces adipogenesis without the adipogenesis-inducing DMI medium. Forced expression of MCPIP induces adipogenesis in PPARγ−/− mouse embryonic fibroblasts (MEF). Thus, MCPIP is a newly identified inducer of adipogenesis.

Materials and Methods

Cell Culture

The 3T3-L1 fibroblast cell line was obtained from the American Type Culture Collection (ATCC). PPARγ−/− MEF cell line was obtained from Dr. Bruce Spiegelman, Harvard University. Cells were maintained in Dubelco's Modified Eagles Medium (DMEM) with 1% penicillin/streptomycin and 10% FBS until experimentation. Cells growing on plates at 60% confluency were treated with or without 10ng/mL MCP-1 for 7 days. Adipocyte differentiation was induced by replacing DMEM with media containing DMI (Zen-Bio, Inc). Construction of the MCPIP-GFP expression plasmid has been previously described [8]. Cells were transfected with 100nmol/L of a chemically synthesized siRNA targeted for MCPIP (Ambion) or with100nmol/L non-specific siRNA (Ambion) 12 hr prior
to treatment with DMI. All cells types were transfected using FuGENE HD (Roche
Applied Science).

Oil Red-O

3T3-L1 or PPARγ−/− MEF cells were fixed with 10% formalin for 30min at room
temperature 8 days after experimental treatment. Cells were then washed with 60%
isopropanol followed by treatment with Oil red-O (2.1mg/mL) for 20min. at room
temperature. Samples were then washed 4 times with H2O. Images of sample plates
stained with Oil red-O were taken and analyzed using morphometric software analysis.
Oil red-O was evaluated by measuring total objects stained, area of plate stained and
total intensity of stained area. All three methods yielded similar results. After examining
the plates microscopically, they were treated with 100% isopropanol to extract Oil red-
O. The solution was then measured for absorbance at 520nm.

RT-PCR

Total RNA was isolated from treated fibroblasts using TRIzol (Invitrogen). First-strand
cDNA was synthesized using 1 μg total RNA (DNase-treated) using Applied
Biosystem’s High Capacity RT cDNA synthesis kit. β-actin served as internal control.
The primer set F-5'-ctccaatgttctcaaaacttac-3’ and R-5’-gtattcctatggcttccagtgc-3’ was used
to detect a 400bp product in the PPARγ−/− MEF cell line to confirm the deletion of exon 2
in PPARγ (9).
Immunoblot Analysis

3T3-L1 and PPARγ−/− MEFs were treated with cell lyses buffer (20% glycerol 0.1% TritonX 8% 0.5M EDTA and 1% 1M DTT) and protein samples were collected and subjected to immunoblot using polyclonal antibodies specific for MCPIP (1:2000), C/EBPα (1:2000), C/EBPβ (1:2000, Abcam), C/EBPδ (1:2000, Abcam), PPARγ (1:2000, Abcam), Adiponectin (1:3000, Abcam), LPL (1:2000, Abcam), aP2 (1:2000, Abcam), and Apelin (1:3000, Abcam). Rabbit secondary antibody was used on all at a concentration of 1:5000 except aP2 which used a chicken secondary antibody at a concentration of 1:5000).

Results

We recently reported that MCP-1 binding to CCR2 leads to the induction of a novel zinc finger protein, called MCPIP, that can induce cell death [8] or differentiation leading to angiogenesis [10]. MCP-1 production by preadipocytes has been detected [11] and in a tissue engineering model of adipogenesis MCP-1 treatment was reported to increase adipose tissue mass in vivo [9]. In view of these observations that suggest the possibility of a role for MCP-1 in adipogenesis, we tested whether differentiation of 3T3-L1 cells into adipocytes involves MCP-1. First, we tested whether 3T3-L1 cells were induced to produce MCP-1 by DMI medium. Real time RT-PCR showed that MCP-1 transcript level reached maxima 4hr after treatment with DMI medium compared to non-DMI containing medium (Fig. 20A). We then tested whether MCP-1 treatment could induce differentiation of 3T3L-1 cells into adipocytes in a non-DMI containing medium.
Figure 20: 3T3-L1 fibroblasts produced MCP-1 when treated with DMI and MCP-1 treatment of 3T3-L1 fibroblasts induced MCPIP expression and adipogenesis.

(A), 3T3-L1 cells were treated with or without DMI. RNA was isolated and MCP-1 transcript levels were measured using real time RT-PCR (*=p<0.02). -ΔΔCt values of treated/untreated are displayed. (B), 3T3-L1 cells grown to 60% confluency were treated with or without 10ng/mL of MCP-1 protein for 7 days. RNA and cell lysate were collected 1, 3, 5, and 7 days after treatment. Transcript levels of the adipogenesis markers C/EBPβ, C/EBPδ, C/EBPα, and PPARγ were measured using real time RT-PCR (*=p<0.02). (C), Protein levels of C/EBPβ, C/EBPδ, C/EBPα and PPARγ were measured using immunoblot analysis. Results were normalized against GAPDH (*=p<0.02). (D), RNA and cell lysate were collected 7 days after MCP-1 treatment. Real time RT-PCR was used to measure the adipogenesis markers adiponectin, LPL and AP2 (normalized to β-actin, -ΔΔCt values of treated/untreated are displayed *=p<0.02). (E), Protein levels of adiponectin, LPL and AP2 were measured using immunoblot analysis. Results were normalized against GAPDH (*=p<0.02). (F), Cells were stained with oil red O 8 days after MCP-1 treatment. (G), MCPIP transcript levels were measured using real time RT-PCR (normalized to β-actin, -ΔΔCt values of treated/untreated are displayed). Protein levels of MCPIP were measured using immunoblot analysis. Results were normalized against GAPDH (*=p<0.02). All experiments were repeated three times.
MCP-1 treatment induced expression of the C/EBP family of transcription factors and PPARγ that is known to be induced during differentiation of 3T3-L1 cells into adipocytes (Fig. 20B). C/EBPβ and C/EBPδ were induced early followed by induction of C/EBPα and PPARγ. Induction of these adipogenesis-associated transcription factors was clearly seen at the transcript level determined by real time PCR analysis and at the protein level as determined by immunoblot analysis (Fig. 20B, C). MCP-1 treatment induced adipocyte markers. Induction of adiponectin, LPL, and AP2 was clearly seen at the transcript level determined by real time PCR and protein level as determined by immunoblot analysis (Fig. 20D, E). Microscopic examination revealed lipid body accumulation. Oil red O staining revealed robust accumulation of lipid bodies (Fig. 20F). We tested whether MCP-1 induced adipogenesis is mediated via MCPIP. Upon MCP-1 treatment, MCPIP was induced to a maximal level during the first day (Fig. 20G). This induction was clear from real time PCR measurement of the transcript level and from the immunoblot analysis of MCPIP protein level. After the second day MCPIP levels decreased. This result is consistent with the involvement of MCPIP in adipogenesis.

If MCPIP is a key player in adipogenesis, it should be induced early during the adipocyte differentiation and induced in 3T3-L1 cells by the commonly used adipogenesis-inducing DMI medium. The levels of MCPIP, C/EBPβ and C/EBPδ reached maximal levels during the first two days. Induction of PPARγ and C/EBPα followed as expected (Fig. 21). That MCPIP plays a critical role in adipogenesis is indicated by the finding that knockdown of MCPIP expression with siRNA specific for MCPIP inhibited adipogenesis induced by DMI medium, whereas nonspecific (scrambled) siRNA had very little effect (Fig. 22A-H).
Figure 21: DMI-induced adipocyte differentiation of 3T3-L1 fibroblast cells elicited an early increase in MCPIP expression.

3T3-L1 fibroblasts were treated with or without DMI. RNA was collected at 1, 2, 3, and 4 days after treatment. Real time RT-PCR was used to measure MCPIP (A), and the known adipogenesis markers C/EBPβ (B), C/EBPδ (C), C/EBPα (D), and PPARγ (E). Results were normalized to β-actin: -ΔΔCt values of treated/untreated are displayed (*=p<0.02, #=p<0.02). (F), Immunoblot analysis was performed with cell lysate collected at the same time points with antibodies against MCPIP, C/EBPβ, C/EBPδ, C/EBPα and PPARγ; (G-K), The results in F were normalized against GAPDH (*=p<0.02, #=p<0.03, t = p<0.05). All experiments were repeated three times.

This inhibition was demonstrated by the inhibition of appearance of adipocyte markers adiponectin and LPL at the transcript level as indicated by real time RT-PCR (Fig. 22A) and at the protein level as measured by immunoblot analysis (Fig. 22B-C). In addition, real time RT-PCR measurements of transcript levels showed that knockdown of MCPIP expression resulted in inhibition of induction of the known adipogenesis-associated transcription factors C/EBPβ, C/EBPδ, C/EBPα and PPARγ (Fig. 22D-G). Staining of the cells with Oil red O after 8 days of differentiation in DMI medium showed that
knockdown of MCPIP by treatment with siRNA, specific for MCPIP, drastically inhibited lipid droplet accumulation, whereas nonspecific siRNA had little effect (Fig. 22H). Measurement of Oil red O extracted from stained cells also shows that knockdown of MCPIP inhibited lipid accumulation (data not shown).

Figure 22: Knockdown of MCPIP inhibited DMI-induced adipogenesis.
3T3-L1 fibroblasts were treated with siRNA specific for MCPIP for 24hr before treatment with DMI. (A), RNA was isolated from all samples 7 days after DMI treatment and was evaluated for transcript levels for the adipocyte markers, adiponectin and LPL by using RT-PCR; β-actin served as a control. (B), Immunoblot analysis was performed with cell lysate collected from all samples 7 days after DMI treatment. The samples were evaluated for the adipocyte markers adiponectin and LPL. (C), The results in B were normalized against GAPDH (* = p<0.02; # = p<0.03). NSsiRNA, nonspecific (scrambled) siRNA. (D-G), RNA was isolated from samples 1, 2, 3 and 4 days after DMI treatment. Real time RT-PCR was used to measure transcript levels of C/EBPβ, C/EBPδ, C/EBPα and PPARγ. β-actin served as a control, -ΔΔCt values of treated/untreated are displayed. (* = p<0.02; # = p<0.03). (H), Cells were stained with Oil Red O 8 days after treatment with DMI. All experiments were repeated three times.
These results suggest that MCPIP plays a critical role in adipogenesis induced by DMI.

If MCPIP is one of the critical regulators involved in adipogenesis, forced expression of MCPIP in 3T3-L1 cells might be expected to cause induction of adipogenesis in the absence of adipogenesis-inducing DMI mixture. Transfection of 3T3-L1 cells with an expression construct for MCPIP-GFP caused the induction of adipogenesis in the absence of DMI medium, whereas GFP controls did not show differentiation (Fig. 23). Forced expression of MCPIP induced the expression of the well-established cascade of gene expression known to occur in DMI medium. MCPIP expression induced C/EBPβ, C/EBPδ, C/EBPα and PPARγ, the transcription factors well known to be involved in adipogenesis. The induction of these transcription factors were seen both at the transcript levels measured by real time RT-PCR (Fig. 23B-E) and at a protein level measured by immunoblot analyses (Fig. 23F-K). C/EBPβ and C/EBPδ, levels reached maximal levels before C/EBPα and PPARγ levels reached maximal levels as seen in DMI medium. MCPIP-induced adipogenesis was demonstrated by the induction of adipocyte markers, adiponectin and LPL, both at the transcript level as indicated by real time RT-PCR and at protein level as measured by immunoblot analysis (Fig. 23L-O). Staining of the cells transfected with MCPIP-GFP expression plasmid, after 8 days in non-DMI medium showed strong staining of lipid bodies whereas GFP controls showed no staining (Fig. 23P).
Figure 23: Forced expression of MCPIP induced adipogenesis without DMI in 3T3-L1 cells.

3T3-L1 fibroblasts were transfected with MCPIP-GFP or GFP alone. RNA and cell lysate were collected 0.5, 1, 3, and 5 days after transfection. Transcript levels of MCPIP (A), and the adipogenesis markers C/EBPβ (B), C/EBPδ (C), C/EBPα (D), and PPARγ (E), were measured using real time RT-PCR (normalized to β-actin; -ΔΔCt values of MCPIP-GFP/GFP are displayed; * = p<0.02; # = p<0.03; † = p<0.05). (F), Protein levels of MCPIP, C/EBPβ, C/EBPδ, C/EBPα and PPARγ were measured using immunoblot analysis. (G-K). Results in F were normalized against GAPDH (*=p<0.02). RNA and cell lysate were collected 7 days after transfection. (L), Transcript levels of the adipocyte markers, adiponectin and LPL, were measured using RT-PCR; β-actin served as a control. (M), Protein levels of the adipocyte markers, adiponectin and LPL, were measured using immunoblot analysis. (N and O), Results in M were normalized against GAPDH (* = p<0.02). (P), Cells were stained with oil red O 8 days after transfection. All experiments were repeated three times.
Figure 24: Treatment of 3T3-L1 cells with MCP-1 or transfection with MCPIP-GFP resulted in oil droplet accumulation comparable to 3T3-L1 cells treated with the classical cocktail DMI.

3T3-L1 fibroblasts were treated with DMI or MCP-1 or were transfected with MCPIP-GFP. Cells were stained with oil red O 8 days after treatment. Images of plates were captured and staining was measured using morphometric software analysis. Quantitation of lipid accumulation in the entire plate was done on the basis of total stained area, number of cells stained and intensity of staining. All three methods yielded similar results. The results obtained on the basis of total area stained are displayed.

Thus it is clear that MCPIP expression can induce adipogenesis in 3T3-L1 cells in the absence of the usual adipogenesis inducing factors in the medium. MCP-1 treatment and forced expression of MCPIP in non-DMI medium caused accumulation of lipid droplets comparable to that observed in the DMI medium, although the DMI medium induced the most robust degree of lipid droplet accumulation. This conclusion is based on morphometric quantitative analysis of the stained plates (Fig. 24).

In view of the currently held view that adipogenesis cannot be induced in the absence of PPARγ, we tested whether forced expression of MCPIP can induce adipogenesis in PPARγ−/− MEFs. We transfected PPARγ−/− cells with MCPIP-GFP expression plasmids or GFP control and incubated them in DMI medium. Forced expression of MCPIP in PPARγ−/− cells caused induction of the C/EBP family of
transcription factors, C/EBPβ, C/EBPδ, C/EBPα, as seen both at the transcript level as measured by real time PCR (Fig. 25A) and at protein level as measured by immunoblot analysis (Fig. 25 B-E).

Figure 25: Forced expression of MCPIP in PPARγ/- fibroblasts induced adipogenesis.
PPARγ/- fibroblasts were transfected with MCPIP-GFP or GFP alone and were evaluated for adipogenesis. (A), RNA was collected from PPARγ/- fibroblasts 1, 3, and 5 days after transfection with MCPIP-GFP or GFP alone. Transcript levels of C/EBPβ, C/EBP δ, and C/EBPα were evaluated using real-time RT-PCR; β-actin served as a control; -ΔΔCt values of MCPIP-GFP/GFP are displayed (*=p<0.02). (B), Cell lysate was collected from the same samples and were evaluated via immunoblot for C/EBPβ, C/EBPδ, and C/EBPα. (C-E), Results in B were normalized against GAPDH (* = p<0.02; # = p<0.03). (F), Immunoblot analysis was performed with cell lysate collected from all samples 1, 3, 5 and 7 days after transfection. Samples were evaluated for the adipocyte markers AP2, adiponectin, and LPL. (G), The results in A were normalized against GAPDH (*=p<0.02). (H). Cells were treated with Oil Red O 8 days after transfection and were microscopically evaluated. (I), PPARγ +/- or PPARγ/- fibroblasts were transfected with MCPIP-GFP. Samples were evaluated for PPARγ using immunoblot analysis. All experiments were repeated three times.
The MCPIP-GFP transfected cells exhibited adipogenesis as indicated by induction of adipocyte markers, Ap2, adiponectin and LPL, as measured by immunoblot analysis (Fig. 25F and G), whereas GFP controls showed very little induction. Microscopic examination of the Oil Red O stained cells showed robust accumulation of lipid droplets in MCPIP-GFP transfected PPARγ−/− cells whereas GFP controls showed little lipid accumulation (Fig. 25H). Since this result is contrary to the currently accepted view that PPARγ is necessary for adipogenesis, we confirmed that the PPARγ−/− MEFs we used were, in fact, PPARγ−/− as reported by the authors who generated this PPARγ null cell line [12] and as indicated under Material and Methods. Immunoblot analysis confirmed the absence of PPARγ protein (fig. 25 I). Thus, MCPIP expression can induce adipogenesis in the absence of PPARγ.

Discussion

The results presented here constitute the discovery of a previously unknown key player that can induce adipogenesis in the absence of PPARγ that is currently thought to be necessary and sufficient for adipogenesis. The central role of MCPIP in adipogenesis was demonstrated by the finding that MCP-1 production was induced very early after 3T3-L1 cells were placed in DMI medium. Furthermore, MCP-1 treatment of 3T3-L1 cells induced adipogenesis. This direct demonstration of MCP-1 induction of adipogenesis is consistent with a recent report that in vivo, MCP-1 enhanced new adipose tissue formation [9]. Adipogenic precursor cells are known to have CCR2 [13]. Since MCPIP is induced by the signaling process resulting from MCP-1 interaction with its receptor CCR2 [8], the present results have implications on the role of MCP-1/CCR2
in obesity and type 2 diabetes. In diet-induced insulin resistance, *mcp-1* gene expression in adipose tissue increases in ob/ob and db/db mice [14]. Adipocyte-specific over expression of MCP-1 in mice results in increased macrophage recruitment to adipose tissue and causes hepatic steatosis and insulin resistance in liver and muscle as well as fat tissue. In CCR2\(^{-/-}\) mice, recruitment of monocytes /macrophages into adipose tissue is inhibited and development of obesity-induced insulin resistance is attenuated [15]. Macrophage recruitment and consequent inflammatory response in adipose tissue result in secretion of more MCP-1, TNF\(\alpha\) and IL-\(\beta\) [16]. These agents cause increase in lipolysis and decrease in triacylglycerol synthesis leading to increased levels of fatty acids and in the availability of triacylglycerols for uptake directly into skeletal muscle [3]. These excess circulating lipids cause accumulation of fatty acyl-CoA in skeletal muscle, liver and \(\beta\) cells leading to insulin resistance and type 2 diabetes [17,18]. The inflammatory stress causes IL-6 secretion from the adipose tissue that leads to expression of SOCS3 expression that is known to induce hepatic insulin resistance [19]. Thus, there is strong evidence for a key role that MCP-1/CCR2 system plays in obesity and type 2 diabetes. The role of MCP-1 in this process has been questioned on the basis that macrophage accumulation in adipose tissue was not significantly attenuated in MCP-1 deficient mice [20]. However, MCP-5 can substitute as a CCR2 ligand in mice [21].

The exact source of MCP-1 in adipose tissue is not known although there are several possibilities. Obesity is well known to cause elevation of MCP-1 levels. The resident macrophages and macrophages infiltrating the adipose tissue could produce MCP-1. MCP-1 production could also be caused by endotoxemia from gut flora elevated
by feeding [22]. Death of adipocytes could release MCP-1, as dying cells are known to release MCP-1 [23]. Adipose tissue in obese animals is reported to have localized hypoxia that could lead to HIF induction and MCP-1 production [24, 25]. We have found that under hypoxia human macrophages secrete MCP-1 (unpublished). In adipose tissue macrophage-containing areas were found to be hypoxic and thus these macrophages would also produce MCP-1.

We show that MCP-1 binding to CCR2, that is known to be present in fibroblasts and preadipocytes [13, 26], caused MCPIP induction before C/EBPβ and C/EBPδ were induced. Furthermore, knockdown of MCPIP expression inhibited the induction of these transcription factors and drastically inhibited adipogenesis. Forced expression of MCPIP induced the cascade of transcription factors well known to be induced by DMI medium leading to robust adipogenesis. Thus MCPIP expression induced adipogenesis via the already known sequence of induction of transcription factors and expression of adipogenesis markers. MCPIP induction is one of the earliest events in adipogenesis and MCPIP induced adipogenesis was associated with PPARγ induction. What was surprising was that MCPIP expression could induce adipogenesis in PPARγ−/− cells in which no other factor has previously been able to induce adipogenesis. This finding shows that MCPIP is a key regulator of adipogenesis.

How exactly MCPIP induces the expression of the C/EBP family of transcription factors that would lead to adipogenesis remains to be elucidated. Considering the finding that MCPIP is induced before the induction of the C/EBP family, it appears possible that the role of MCPIP is to induce the early transcriptions factors C/EBPβ and C/EBP δ which would then lead to the induction of subsequent factors required for
adipogenesis. C/EBPα and PPARγ can induce each other. However, C/EBPα cannot induce adipogenesis without PPARγ. Our results demonstrate that MCPIP can induce C/EBPα and adipogenesis without PPARγ. Thus, MCPIP can be considered a critical regulator of adipogenesis.

There is overwhelming evidence that demonstrates a key role for CCR2-mediated events in obesity and development of type 2 diabetes. Our results show that MCP-1/CCR2 system plays a direct role in adipogenesis. There is experimental evidence that MCP-1 can promote angiogenesis [27, 28] and that this MCP-1 induced angiogenesis is mediated via MCPIP [10]. Thus, MCPIP induced by MCP-1/CCR2 interaction promotes adipogenesis and angiogenesis to supply blood to growing adipose tissue. These findings help provide a molecular basis for the role of the MCP-1/CCR2 system in the development of obesity and consequent development of type 2 diabetes.
CHAPTER 4: CARDIOMYOYCTE CELL DEATH INDUCED BY HIGH GLUCOSE EXPOSURE IS MEDIATED VIA PRODUCTION AND INDUCTION OF THE ZINC FINGER PROTEIN

Introduction
The number of individuals with diabetes is expected to reach 490 million by the year 2025 [1]. It is well known that individuals with diabetes have an increased risk for developing heart failure [2-4]. Moreover, cardiovascular complications are considered to be the leading cause of mortality in diabetic patients [4, 5]. Diabetes is known to alter cardiac structure and function independent of coronary artery disease and hypertension, a condition known as diabetic cardiomyopathy [4, 6]. Diabetic cardiomyopathy is further defined as a primary disease process that occurs as the result of metabolic insult that leads to myocardial abnormalities and eventual heart failure [3].

There are multiple factors that may contribute to the pathophysiological progression of diabetic cardiomyopathy including autonomic dysfunction, metabolic derangements, abnormalities in ion homeostasis, alteration in structural proteins, and interstitial fibrosis [4, 5, 7]. Recent studies suggest that cardiomyocyte loss also plays a key role in diabetic cardiac damage in both animals and humans [8-12]. Hyperglycemia contributes to cardiomyocyte death and diabetic cardiomyopathy [13]. Hyperglycemia is known to cause apoptosis [9-12] that leads to diabetic cardiomyopathy. Attenuation of hyperglycemia induced cardiomyocyte cell death has been shown to prevent the progression of cardiac complications associated with diabetes [14]. However, the molecular mechanisms by which hyperglycemia contributes to development of diabetic cardiomyopathy remain unclear.
It is well known that inflammation plays a critical role in the pathophysiological progression of cardiovascular diseases and heart failure [15-17]. A role for inflammation in diabetic cardiomyopathy has also been implicated [18]. Experimental evidence obtained from CCR2 deficient mice [19, 20], MCP-1 deficient mice [21, 22], and transgenic mice with cardiac-targeted expression of MCP-1 [23-26] demonstrate a key role for MCP-1 in the development of cardiovascular disease and heart failure. Interestingly, increased levels of serum MCP-1 have been found in humans to correlate with markers of the metabolic syndrome, including obesity, insulin resistance, type 2 diabetes, hypertension, and increased serum TG concentration [27] Furthermore, endothelial cells and monocytes exposed to high-glucose levels are known to produce MCP-1 [28]. We recently reported that MCP-1 induces a novel zinc-finger transcription factor, termed MCPIP [29]. The presence of MCPIP protein and transcripts has been found associated with apoptotic cardiomyocytes in a murine model of heart failure. Association of MCPIP with ischemic heart disease was also found in the human disease [29]. If and how glucose-induced MCP-1 and MCPIP might play a role in the pathophysiological progression of diabetic cardiomyopathy remains unknown.

In this report we provide evidence that exposure of H9c2 cardiomyoblasts to high glucose causes increased production of MCP-1 and expression of MCPIP. We show that glucose induced cardiomyoblast cell death is mediated via induction of MCPIP caused by MCP-1 production. Moreover, H9c2 cells exposed to high glucose levels display increased ROS production that results in an ER stress response that leads to autophagy and H9c2 cardiomyoblast cell death. We found that MCP-1 induction of MCPIP was responsible for the mediation of this series of events that led to cell death.
Furthermore, the key findings observed in H9c2 cardiomyoblasts were further validated in isolated neonatal rat cardiomyocytes. Thus, our results show that hyperglycemia-induced MCP-1 production and the consequent induction of MCPIP leads to the pathophysiological progression of diabetic cardiomyopathy.

Materials and Methods

Neonatal Rat Cardiomyocyte Isolation

Neonatal rat ventricular myocytes (NRVM) were isolated from hearts of 2-3 day-old Sprague-Dawley rats by Trypsin digestions as described [30, 31]. In brief, hearts were removed surgically and ventricular cardiomyocytes were prepared by 0.12% Trypsin (Invitrogen) in Calcium-free phosphate-buffered saline (PBS: 137 mM NaCl, 2.68 mM KCl, 10 mM Na2HPO4, 1.76 mM KH2PO4, pH 7.4), Cardiomyocytes were preplated for 2 hrs in DMEM supplemented with 15% FBS containing appropriate antibiotics to reduce non-myocyte contamination and then plated (2.0 x 10^6 cells) in culture flasks and incubated at 37 C and 5% CO2 in humidified atmosphere.

Cell Culture

H9c2 rat cardiomyoblasts (ATCC) were grown in Dubelco’s Modified Eagles Medium (DMEM) supplemented with 10% FBS, 4.5g/L L-glucose, 1% penicillin and 1% streptomycin. Cells were treated with or without 28mmol/L D-glucose for 0, 12, 24, or 48hrs. Cells were treated with or without 50μmol/L 3’-methyladenine; 1μmol/L LY294002; 100μmol/L tauroursodeoxycholate (TUDC); 50μmol/L 4-phenylbutiric acid
(4-PBA); 50μmol/L L-NAME; 100nmol/L CeO2, or 20μmol/L apocynin starting 3hrs prior to treatment with 28mol/L glucose. Cell viability and death were measured by (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), trypan blue and TUNEL assays using standard procedures.

ROS Measurements

ROS production in H9c2 cardiomyoblasts was measured 24 hrs after treatment with 28mM glucose using 1μmol/L Dihydrorhodamine 123 (DHR123) for 30min. at 37 C and 5%CO2 and were evaluated with a flurometric plate reader (Excitation: 550nm and Emission:590nm).

RT-PCR

Total RNA was isolated with the RNAeasy kit (Invitrogen) from H9c2 cardiomyoblasts treated with or without 28mmol/L glucose and first-strand cDNA was synthesized using 1μg total RNA (DNase-treated) using I script cDNA synthesis kit (Bio-Rad); β-actin served as an internal control. Primers designed for real-time PCR using primer quest were:

- MCPIP: F-5’-TGTGCCTATCACAGACCAGCACAT-3’; R-5’-TCGGATTCCATAGGCCAGCTTCACA-3’
- GRP78: F-5’-AGCCCACCGTAACAATCAAGGTCT-3’; R-5’-CGTGTAATGCGCTCTTTGA GCTT-3’
- PDI: F-5’-CAGAATGGAAACCAGCACAAACCA-3’; R-5’-GCCACATCCACCACATCAAACAA-3’
**Immunoblot Analysis**

H9c2 cardiomyoblasts were treated with cell lysis buffer (20% glycerol 0.1% TritonX 8% 0.5M EDTA and 1% 1M DTT) and protein samples were collected and subjected to immunoblot using the following polyclonal antibodies: anti-MCPIP (1:2000); anti-BECN1 (Santa Cruz; 1:500); anti-LC3 ; anti-GRP78 (1:2000) ; anti-PDI (1:2000) anti-phox47 (1:1000); and anti-iNOS (Upstate; 1:2000). Immunoblots were quantified as a ratio over β-actin expression.

**siRNA Treatment**

H9c2 cardiomyoblasts were treated with 100nmol/L of a chemically synthesized siRNA targeted for MCPIP, BECN1, or IRE1 (Ambion) or with 100nmol/L non-specific siRNA (Ambion) using Dharmafect transfection reagent 12hrs prior to treatment with or without 28mM glucose.

**Statistical Analysis:**

The experimental data was analyzed by using SPSS statistical software (SPSS Inc.) under Windows XP. All values are presented as mean ± SEM. Results were compared between groups by ANOVA analysis followed by t tests. Differences were considered significant at a p value of <0.05.
Results

High glucose induced death of H9c2 cardiomyoblasts is mediated via MCP-1 production and induction of MCPIP.

Since it is known that hyperglycemic conditions can induce the expression of MCP-1 in cultured monocytes [27], we tested whether H9c2 cardiomyoblasts exposed to high glucose (28mmol/L glucose) concentration could also cause production of MCP-1. We found that MCP-1 protein levels significantly increased in H9c2 cardiomyoblast 12hrs after treatment with 28mmol/L glucose and reached maximal levels at 24hrs after treatment (Fig. 26A). To determine whether the MCP-1 produced by H9c2 cardiomyoblasts treated with 28mmol/L is involved in cell death induced by high glucose exposure, we tested whether inhibition of MCP-1 binding to its CCR2 receptor would attenuate high glucose induced cell death in H9c2 cardiomyoblasts. Antibody specific to CCR2 significantly attenuated glucose-induced H9c2 cardiomyoblast cell death (28mmol/L = 31± 1%; anti-CCR2 = 11± 2%; Fig. 26B). G-protein coupled inhibitors propagermanium and pertussis toxin (PTX) also attenuated high-glucose induced cell death (28mmol/L = 31± 1%; propagermanium = 12±1%; PTX = 11±2%; Fig. 26B). These results indicate that MCP-1 binding to its receptor mediates H9c2 cardiomyoblast death induced by high glucose treatment.

Since we have previously shown that MCP-1 can induce MCPIP [29], we tested whether 28mmol/L glucose treatment of H9c2 cardiomyoblasts could induce MCPIP. The glucose treatment induced MCPIP production (Fig. 26C). That the MCPIP induction was mediated via MCP-1 interaction with CCR2 was shown by the fact that treatment
with CCR2 antibody, and the G-protein coupled receptor inhibitors propagermanium and pertussis toxin attenuated induction of MCPIP protein levels by high glucose treatment of H9c2 cardiomyoblasts (Fig. 26D).

Figure 26: H9c2 cardiomyoblasts treated with 28mmol/L glucose resulted in MCP-1 production and MCPIP.

H9c2 cardiomyoblasts were treated with or without 28mmol/L glucose. (A), At 0, 12, and 24 hrs, cell lysate was collected and analyzed using immunoblot with MCP-1 antibody. The results were quantified against β-actin (*=p<0.03). (B), H9c2 cardiomyoblasts treated with 28mmol/L glucose were treated with or without 25ug/mL CCR2 antibody, 2ug/mL propagermanium, or 250ng/mL pertussis toxin (PTX) for 24 and 48 hrs. Cells were then evaluated for cell death using trypan blue (*=p<0.03). (C), Cell lysate was collected at 24 and 48 hrs. and analyzed using immunoblot with MCPIP antibody. β-actin served as a control. (D), H9c2 cardiomyoblasts treated with 28mmol/L glucose were treated with or without 25ug/mL CCR2 antibody (lane 2), 2ug/mL propagermanium (lane 3), or 250ng/mL pertussis toxin (PTX) (lane 4) for 24hrs. Control = lane 1. Cell lysate was collected and analyzed using immunoblot with MCPIP antibody. β-actin served as a control.
To determine if MCPIP is involved in H9c2 cardiomyoblast death induced by high glucose exposure, we tested whether knockdown of MCPIP could attenuate this cell death. We found that knockdown of MCPIP with MCPIP specific siRNA resulted in the attenuation of H9c2 cardiomyoblast death induced by high glucose exposure while treatment with non-specific siRNA had little effect (Fig.27). These results demonstrate that H9c2 cardiomyoblast death induced by high glucose exposure is mediated via MCP-1 induction of MCPIP.

**Figure 27:** H9c2 cardiomyoblasts treated with 28mmol/L glucose resulted in cell death that was mediated via MCPIP.

H9c2 cardiomyoblasts were treated with 28mmol/L glucose with or without siRNA specific for MCPIP or with non-specific siRNA. (A), Cell lysate was collected and analyzed using immunoblot with MCPIP antibody at 24hrs. β-actin served as a control (#=p<0.02). (B), At 24 and 48 hrs, cell death was analyzed using trypan blue or TUNEL assay (C, *=p<0.03).
H9c2 cardiomyoblasts exposed to high glucose levels resulted in ROS production that was mediated via MCPIP

Since cardiomyocytes treated with high glucose concentrations have been shown to produce increased levels of reactive oxygen species (ROS) [8, 12], we tested whether MCPIP plays a role in the production of ROS in H9c2 cardiomyoblasts exposed to high glucose levels. H9c2 cardiomyoblasts exposed to 28mmol/L glucose resulted in an increase in fluorescence of the free radical dye DHR123 24hrs. post treatment (Fig.28A). Knockdown of MCPIP with specific siRNA significantly attenuated ROS production as compared to untreated and non-specific siRNA controls (Fig.28A). NADPH oxidase activation is considered to be a major source of ROS production in various cell lines treated with high glucose [12]. We tested whether MCPIP mediated ROS production in H9c2 cardiomyoblasts exposed to high glucose occurs via an increase in NADPH oxidase activity. Our results showed that H9c2 cardiomyoblasts treated with 28mmol/L glucose resulted in the migration of the NADPH oxidase subunit phox47 to the plasma membrane (Fig.28B), indicating activation of phox47, while knockdown of MCPIP with specific siRNA showed no migration to the membrane fraction (Fig.28C). Thus, in H9c2 cardiomyoblasts exposed to high glucose levels, MCPIP mediates the activation of NADPH oxidase resulting in the increased production of ROS. This notion is further supported by our observation that treatment of H9c2 cells with the ROS/RNS inhibitors apocynin, CeO2 and L-NAME prior to treatment with 28mmol/L glucose resulted in the attenuation of ROS production as detected by DHR123 (Fig. 28D).
Figure 28: H9c2 cardiomyoblasts treated with 28mmol/L glucose resulted in cell death that was mediated via MCPIP induced ROS production.

(A), H9c2 cardiomyoblasts were treated with 28mmol/L glucose with or without siRNA specific for MCPIP or with non-specific siRNA. ROS was then measured using DHR 123 (excitation: 550nm; emission 590nm, \( t=p<0.05 \)). (B), H9c2 cardiomyoblasts were treated with or without 28mmol/L glucose. Cell lysate was collected and the membrane fractions were isolated. Both total lysate and the membrane fraction were evaluated using immunoblot with phox47 antibody. Results were quantified by taking the membrane fraction/total lysate (*=p<0.03). (C), H9c2 cardiomyoblasts were treated with 28mmol/L glucose with or without siRNA specific for MCPIP or with non-specific siRNA. Cell lysate was collected and the membrane fractions were isolated. Both total lysate and the membrane fraction were evaluated using immunoblot with phox47 antibody. Results were quantified by taking the membrane fraction/total lysate (*=p<0.03).(D), H9c2 cardiomyoblasts treated with 28mmol/L glucose were treated with or without 20\( \mu \)M Apocynin, 300nM CeO2, or 50\( \mu \)M L-NAME. At 0, 24, and 28hrs, ROS was measured using DHR 123 (excitation: 550nm; emission 590nm, (*=p<0.03). At 24 and 48hrs, cells were evaluated for cell death using trypan blue (E) or for cell viability using MTT assay (F) (*=p<0.03).
Since ROS produced by cardiomyocytes under exposure to high glucose levels results in cell death [12], we next tested whether MCPIP induced ROS is involved in high glucose induced H9c2 cardiomyoblast death. We found that treatment with the ROS/RNS inhibitors apocynin, CeO2 and L-NAME resulted in the attenuation of high glucose induced H9c2 cardiomyoblast cell death as detected by trypan blue (Fig. 28E) and caused increased viability in H9c2 cardiomyoblasts as determined by MTT assay (Fig. 28F). Thus, our results indicate that high glucose-induced H9c2 cardiomyoblast death occurs via MCPIP-induced oxidative stress.

High glucose treatment of H9c2 cardiomyoblasts resulted in ER stress that was mediated via MCPIP induced ROS production

Since MCP-1 expression in murine hearts is known to induce ER stress in cardiomyocytes [32], we tested whether exposure of H9c2 cardiomyoblasts to high glucose concentrations would result in an increased expression of the ER stress chaperone proteins PDI and GRP78. We found that treatment of H9c2 with 28mmol/L glucose resulted in an increase in both transcript and protein levels of PDI and GRP78 (Fig. 29A and B). We next tested whether knockdown of MCPIP would attenuate high glucose-induced ER stress. We found that knockdown of MCPIP with specific siRNA resulted in significant attenuation of both transcript and proteins levels of GRP78 and PDI as compared to H9c2 cardiomyoblasts treated with non-specific siRNA (Fig. 29C and D). Since ROS is known to induce ER stress [33], we further tested whether MCPIP induced ROS production plays a role in inducing ER stress in H9c2 cardiomyoblasts exposed to high glucose concentrations. We found that treatment of H9c2
cardiomyoblasts with the ROS/RNS inhibitors apocynin and CeO2 prior to treatment with 28mmol/L glucose resulted in the attenuation of PDI and GRP78 protein levels. This result suggests that MCPIP induced ROS production does cause ER stress in H9c2 cardiomyoblasts treated with 28mmol/L glucose.

Figure 29: H9c2 cardiomyoblasts treated with 28mmol/L glucose resulted in MCPIP induced ER stress.
(A), H9c2 cardiomyoblasts were treated with or without 28mmol/L glucose. RNA was isolated at 24 and 48 hrs. Transcript levels of PDI (left) and GRP78 (right) were evaluated using real-time RT-PCR. The results are expressed as -ΔΔCT as was calculated by ΔCT treated/ΔCT untreated. ΔCT values were calculated using β-actin controls. (B), Cell lysate was collected from the same samples and was evaluated using immunoblot with antibody for PDI or GRP78. (C), H9c2 cardiomyoblasts were treated with 28mmol/L glucose with or without siRNA specific for MCPIP or with non-specific siRNA. RNA was isolated at 24 hrs. Transcript levels of MCPIP were evaluated using real-time RT-PCR. The results are expressed as -ΔΔCT as was calculated by ΔCT treated/ΔCT untreated. ΔCT values were calculated using β-actin controls (*=p<0.03). (D), Cell lysate was collected from the same samples and was evaluated using immunoblot with antibody for PDI or GRP78. (E), H9c2 cardiomyoblasts treated with 28mmol/L glucose were treated with or without 20µM Apocynin, 300nM CeO2, or 50µM TUDC. At 24 hrs, cell lysate was collected and analyzed using immunoblot with PDI or GRP78 antibody.
Figure 30: H9c2 cardiomyoblasts treated with 28mmol/L glucose resulted in cell death that was mediated via MCPIP induced ER stress. (A), H9c2 cardiomyoblasts treated with 28mmol/L glucose were also treated with or without 100µM TUDC or 50µM 4-PBA. Cells were then evaluated for cell death using trypan blue ($t=p<0.05$). (B), Cell viability of the same samples was also evaluated using MTT assay ($*=p<0.03$). (C-D), H9c2 cardiomyoblasts were treated with 28mmol/L glucose with or without siRNA specific for IRE1 or with non-specific siRNA. Cell death was evaluated using trypan blue.

ER stress is known to lead to signaling events that can cause cell death [34-36]. We tested whether chemical chaperones that are known to inhibit ER stress [37] could prevent high glucose induced H9c2 cardiomyoblast cell death. We found that inhibition of ER stress with TUDC and 4-PBA resulted in the attenuation of H9c2 cardiomyoblast cell death as determined by trypan blue (Fig.30A) and resulted in increased cell viability as detected by MTT (Fig 30B). Since IRE1 is an ER stress protein that is known to signal cell death [34, 38, 39], we tested whether knockdown of IRE1 could prevent high glucose induced H9c2 cardiomyoblast death. Knockdown of IRE1 significantly
attenuated high glucose-induced cardiomyoblast death (Fig. 30C and D). Thus, high glucose treatment of H9c2 cardiomyoblasts results in the induction of MCPIP that leads to ER stress that causes cell death.

High glucose treatment of H9c2 cardiomyoblasts resulted in the induction of autophagy that occurred via MCPIP induced ER stress

Since ER stress is known to induce autophagy that has been shown to be involved in cardiomyocyte cell death involved with heart failure [40-42], we tested whether high glucose induction of MCPIP results in autophagy that is mediated via ER stress. We found that treatment of H9c2 cardiomyoblasts with 28mmol/L glucose resulted in an increase in the autophagy marker beclin-1 (Fig. 31A). Furthermore, knockdown of MCPIP with specific siRNA resulted in the attenuation of beclin-1 expression caused by glucose treatment (Fig. 31B). This result was further supported by the observation that H9c2 cardiomyoblasts treated with 28mmol/L glucose resulted in cleavage of LC3 that is indicative of autophagy (Fig. 31A). Knockdown of MCPIP with specific siRNA prevented the cleavage of LC3I (Fig. 31B). To test whether the ER stress induced by MCPIP was responsible for the observed autophagy caused by treatment with 28mmol/L glucose, we tested whether inhibition of ER stress with the specific chemical chaperones TUDC and 4-PBA [37] would prevent induction of beclin-1. Treatment with TUDC and 4-PBA prior to treatment with 28mmol/L glucose resulted in the attenuation of beclin-1 protein levels (Fig. 31C). These results indicate that high glucose induced autophagy occurs via MCPIP induction of ER stress.
Since autophagy is known to cause cell death [43, 44], and since a role for autophagy in cardiomyocyte cell death involved with heart failure has been implicated [40-42, 45], we tested whether high glucose induced cell death is mediated via MCPIP induction of autophagy. Knockdown of beclin-1 and treatment with the autophagy inhibitors 3’MA and LY294002 resulted in the attenuation of high glucose-induced cell death as detected by trypan blue (Fig.32A-C).

Figure 31: H9c2 cardiomyoblasts treated with 28mmol/L glucose resulted in autophagy that was mediated via MCPIP induced ER stress. H9c2 cardiomyoblasts were treated with or without 28mmol/L glucose. (A), At 24 and 48 hrs., cell lysate was collected and analyzed using immunoblot with beclin-1 or LC3 antibody. β-actin served as a control. (B), H9c2 cardiomyoblasts were treated with 28mmol/L glucose with or without siRNA specific for MCPIP or with non-specific siRNA. At 48 hrs., cell lysate was collected and analyzed using immunoblot with beclin-1 or LC3 antibody. β-actin served as a control. (C), H9c2 cardiomyoblasts treated with 28mmol/L glucose were treated with 100µM TUDC or with 50µm 4-PBA. At 48 hrs, cell lysate was collected and analyzed using immunoblot with antibody specific for beclin-1. β-actin served as a control.

These results show that treatment of H9c2 cardiomyoblasts with high glucose concentration results in the induction of MCPIP that leads to ROS production that results in ER stress that causes autophagy and the induction of cell death.
Figure 32: MCPIP induced autophagy mediates high glucose induced H9c2 cardiomyoblast death.

(A-B), H9c2 cardiomyoblasts were treated with 28mmol/L glucose with or without siRNA specific for beclin-1 or with non-specific siRNA. At 24 and 48hrs after treatment, cell death was analyzed using trypan blue (*=p<0.05). (C), H9c2 cardiomyoblasts treated with 28mmol/L glucose were treated with or without 50µM 3'MA or 1µM LY294002. At 24 and 48hrs after treatment, cell death was analyzed using trypan blue (#=p<0.03).

The molecular mechanisms of high glucose-induced cardiomyocyte cell death were similar to those observed in high glucose treated H9c2 cardiomyoblasts.

To test whether the molecular events that lead to glucose-induced death of H9c2 cardiomyoblasts also occur in real cardiomyocytes, we examined the glucose-induced events in neonatal rat ventricular cardiomyocytes. We found that cardiomyocytes treated with 28mmol/L glucose resulted in increased MCP-1 protein expression (Fig. 33A). Furthermore, inhibition of MCP-1’s interaction with CCR2 with antibody for CCR2, and the G-coupled protein receptor inhibitors propagermanium and pertussis toxin resulted in the attenuation of glucose-induced cardiomyocyte death (Fig. 33B). High glucose treatment of cardiomyocytes resulted in induction of MCPIP while treatment of NRVM’s with CCR2 antibody, propagermanium and pertussis toxin attenuated MCPIP.
induction (Fig. 33C). The involvement of MCPIP in glucose-induced cell death was confirmed as knockdown of MCPIP with specific siRNA resulted in the attenuation of glucose-induced cardiomyocyte death (Fig. 33D).

Glucose-induced ROS/RNS production in cardiomyocytes as measured with DHR 123 was attenuated by knockdown of MCPIP with specific siRNA (Fig. 34A). Glucose induction of ER stress was mediated via MCPIP in cardiomyocytes as knockdown of MCPIP with siRNA resulted in the attenuation in the protein expression of the ER chaperone GRP78. In fact, MCPIP knockdown with siRNA virtually eliminated GRP78 (Fig. 34B). Treatment with the ER stress specific inhibitor TUDC resulted in the attenuation of glucose induced cell death in cardiomyocytes as determined by trypan blue (Fig. 34C). High glucose-induced cell death in cardiomyocytes was found to be mediated via MCPIP induction of autophagy. Cardiomyocytes treated with high glucose resulted in increased levels of the autophagy marker protein beclin-1 while knockdown of MCPIP with siRNA prevented its expression (Fig. 35A). Moreover, treatment of NRVM’s with 28mmol/L glucose and the autophagy inhibitor LY294002 resulted in the attenuation of glucose induced NRVM death as determined by trypan blue (Fig. 35B). These results strongly suggest that high glucose-induced cardiomyocyte death occurs via production of MCP-1 and induction of MCPIP that results in ROS production that leads to ER stress that causes autophagy and eventual cell death.
Figure 33: Cardiomyocytes treated with 28mmol/L glucose resulted in MCP-1 production and MCPIP induction that mediated high glucose induced cell death.

Cardiomyocytes were treated with or without 28mmol/L glucose. (A), At 12, and 24 hrs. cell lysate was collected and analyzed using immunoblot with MCP-1 antibody. The results were quantified against β-actin (*=p<0.03). (B), Cardiomyocytes treated with 28mmol/L glucose were treated with or without 25ug/mL CCR2 antibody, 2ug/mL propagermanium, or 250ng/mL pertussis toxin (PTX) for 24 and 48 hrs. Cells were then evaluated for cell death using trypan blue. (C), Cell lysate was collected at 24 and 48 hrs. and analyzed using immunoblot with MCPIP antibody. β-actin served as a control. H9c2 cardiomyoblasts treated with 28mmol/L glucose were treated with or without 25ug/mL CCR2 antibody (Lane 2), 2ug/mL propagermanium (Lane 3), or 250ng/mL pertussis toxin (PTX) (Lane 4) for 24hrs. Lane 1 is without inhibitor. Cell lysate was collected and analyzed using immunoblot with MCPIP antibody. β-actin served as a control. (D), Cardiomyocytes were treated with 28mmol/L glucose with or without siRNA specific for MCPIP or with non-specific siRNA. At 48 hrs, cell death was analyzed using TUNEL assay (*=p<0.03).
Figure 34: Cardiomyocytes treated with 28mmol/L glucose resulted in cell death that was mediated via MCPIP induced ROS production and ER stress. 

(A), Cardiomyocytes were treated with 28mmol/L glucose with or without siRNA specific for MCPIP or with non-specific siRNA. ROS was then measured using DHR 123 (excitation: 550nm; emission 590nm; *=p<0.03).

(B), Cardiomyocytes were treated with 28mmol/L glucose with or without siRNA specific for MCPIP and non-specific siRNA or with or without 100µM TUDC. At 48 hrs, cell lysate was collected and analyzed using immunoblot with beclin-1. β-actin served as a control.

(C), Cardiomyocytes treated with 28mmol/L glucose were also treated with or without 100µM TUDC. At 48hrs., cells were then evaluated for cell death using trypan blue (#=p<0.02).
Figure 35: MCPIP induced autophagy mediates high glucose induced cardiomyocyte death. (A-B), Cardiomyocytes were treated with 28mmol/L glucose with or without siRNA specific for beclin-1 or with non-specific siRNA. At 48hrs after treatment, cell death was analyzed using trypan blue (*=p<0.03).

Discussion

Hyperglycemia is known to cause cardiomyocyte apoptosis that plays a key role in the pathophysiological development of diabetic cardiomyopathy [8-13]. However the molecular mechanisms by which hyperglycemia induces cardiomyocyte apoptosis are not fully understood. Here we report that hyperglycemia induces MCP-1 production in H9c2 cardiomyoblasts and neonatal cardiomyocytes that leads to the induction of the zinc-finger protein MCPIP which mediates high-glucose induced H9c2 cardiomyoblast death. Furthermore, our study revealed that high glucose-induced H9c2 cardiomyoblast and cardiomyocyte death was mediated via MCPIP induction of ROS that led to ER stress that caused autophagy and eventual cell death.
Diabetic cardiomyopathy is a major complication associated with diabetes mellitus [1-5]. Diabetic cardiomyopathy differs from heart failure caused by coronary heart disease in that it can occur independent of atherosclerosis and hypertension [4, 6]. Inhibition of hyperglycemic conditions has been shown to attenuate the cardiovascular complications associated with diabetes mellitus [14]. Furthermore, cardiomyocytes exposed to hyperglycemic conditions are known to undergo apoptosis [8-12]. Our results are consistent with these observations and provide a molecular explanation into how hyperglycemia can result in cardiomyocyte cell death involved with diabetic cardiomyopathy.

MCP-1 is well known to be a critical player in the pathophysiological progression of heart failure attributed to coronary artery disease [19-26]. Diabetes is associated with elevated MCP-1 levels. Our results demonstrate that MCP-1 may also play a role in the development of diabetic cardiomyopathy. Increased MCP-1 expression has been found in STZ induced diabetic animal hearts [46]. Moreover, MCP-1 serum levels are increased in diabetic individuals and expression of CCR2 on monocytes is known to be elevated in diabetic patients [47]. Our results showed that MCP-1 is produced and H9c2 cardiomyoblasts and cardiomyocytes treated with high glucose concentrations. This is consistent with a previous report showing that high glucose treatment of monocytes results in MCP-1 production [28]. Our results suggest MCP-1 as a critical player in glucose-induced death has inhibition of MCP-1’s interaction with CCR2 attenuated high glucose-induced H9c2 cardiomyoblast and cardiomyocyte death. We have previously shown that MCP-1 can induce cardiomyocyte apoptosis via induction of the zinc-finger protein, MCPIP [29]. Our finding that knockdown of MCPIP in H9c2 cardiomyoblasts
and cardiomyocytes attenuated high glucose-induced cell death supports a role for MCPIP as a key mediator of cardiomyocyte loss that leads to diabetic cardiomyopathy. Increased ROS production is observed in both type 1 and type 2 diabetes and is considered to be a major contributing factor in the development of diabetic cardiomyopathy [48-50]. There are several reports demonstrating a role for ROS in glucose-induced cardiomyocyte cell death [8, 12]. Consistent with these findings, we showed that high glucose treatment of H9c2 cardiomyoblasts and cardiomyocytes induced ROS production. Our results demonstrated that MCPIP was responsible for this increase in ROS production. NADPH oxidase has been shown to be a major source of ROS production in cardiomyocytes treated with high glucose levels [11, 12]. Our results demonstrate that MCPIP mediates glucose-induced NADPH oxidase activation. CeO2 nanoparticles act as free radical scavengers due to their dual oxidative state as the loss of oxygen and the reduction of Ce$^{4+}$ to Ce$^{3+}$ results in the creation of an oxygen vacancy. CeO$_2$ nanoparticles have been utilized in biological systems to mediate oxidative stress-induced cell death [51]. Here we showed that CeO$_2$ nanoparticles attenuate glucose-induced ROS production and cell death. That inhibition of MCPIP induced ROS production was found to attenuate high-glucose induced H9c2 cardiomyoblast death places MCPIP as a key regulator of hyperglycemia-induced ROS production that leads to cardiomyocyte death.

Oxidative stress is known to lead to an accumulation of unfolded proteins and an ER stress response [33, 52, 53]. Major chaperone proteins involved in ER stress include GRP78 and PDI [33, 52, 53]. Our results showed that both GRP78 and PDI increased in H9c2 cardiomyoblasts and cardiomyocytes treated with high glucose levels. This high
glucose-induced ER stress response was mediated via MCPIP as knockdown of MCPIP attenuated the expression levels of GRP78 and PDI. CeO$_2$ nanoparticles have been previously shown to provide a cardioprotective effect through the attenuation of ER stress in a MCP-1 transgenic model of ischemic heart disease (2). Here we showed that CeO$_2$ nanoparticles attenuated MCPIP mediated ER stress induced by high glucose. These results demonstrate a role for ER stress in high glucose-induced cardiomyocyte death that contributes to the pathophysiological progression of diabetic cardiomyopathy. Prolonged ER stress is known to result in signaling events known as the unfolded protein response that eventually leads to cell death [34, 38, 39]. IRE1 is a major signaling protein involved in ER stress induced apoptosis [34, 38, 39]. IRE1alpha is known to induce JNK activation that in turn can result in an increase in pro-apoptotic proteins such as p53 and PUMA [34, 38]. Our results show that MCPIP induced ER stress is involved in cardiomyocyte death induced by exposure to high glucose concentration. This was evident as inhibition of ER stress with the chemical chaperones TUDC and 4’PBA [37] resulted in the attenuation of glucose-induced cell death. Furthermore, knockdown of IRE1 attenuated high glucose-induced H9c2 cardiomyoblast death demonstrating the involvement of ER stress signaling events in high glucose-induced cell death. Thus our results demonstrate that MCPIP-induced ER stress and its signaling events play an important role in high glucose-induced H9c2 cardiomyoblast and cardiomyocyte death.

Prolonged ER stress is known to cause the induction of autophagy that is known to be involved in cardiovascular diseases [40-42]. Although autophagy is thought to at first protect the cell from increased stress [54, 55], it is known that prolonged autophagy can
lead to cell death [43]. Autophagy signaling events depend on several key proteins that orchestrate the formation of the double membrane bound autophagosome that is a hallmark of autophagy. Beclin-1 is required to interact with PI3K II and this results in the eventual cleavage of LC3 that is required for assembly of the autophagosome [36, 56]. Our results indicated that autophagy was involved in H9c2 cardiomyoblast and cardiomyocyte death induced by exposure to high glucose concentrations as an increase in protein levels of beclin-1 and a significant increase in LC3 cleavage product was observed in glucose treated cells. That knockdown of MCPIP in H9c2 cardiomyoblasts treated with high glucose concentrations resulted in the attenuation of both beclin-1 and LC3 cleavage demonstrates that MCPIP mediates this event. Moreover, our finding that inhibition of autophagy with specific inhibitors and via knockdown of beclin-1 attenuated high glucose-induced H9c2 cardiomyoblast and cardiomyocyte death establishes the involvement of MCPIP induced autophagy in glucose-induced cell death.

Our results link hyperglycemia to MCP-1 production of MCPIP induction that provides a molecular linkage between inflammation and diabetic cardiomyopathy. The involvement of MCP-1 and MCPIP in high glucose induced cell death provides a molecular mechanism that can be used as potential therapeutic targets in the treatment and prevention of heart failure that occurs in diabetic individuals.
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Chapter 1


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Chapter 2:


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