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Arsenite Activates NFκB Through Induction of C-Reactive Protein

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Abstract

C-Reactive protein (CRP) is an acute phase protein in humans. Elevated levels of CRP are produced in response to inflammatory cytokines and are associated with atherosclerosis, hypertension, cardiovascular disease and insulin resistance. Exposure to inorganic arsenic, a common environmental toxicant, also produces cardiovascular disorders, namely atherosclerosis and is associated with insulin-resistance. Inorganic arsenic has been shown to contribute to cardiac toxicities through production of reactive oxygen species (ROS) that result in the activation of NFκB. In this study we show that exposure of the hepatic cell line, HepG2, to environmentally relevant levels of arsenite (0.13 to 2 μM) results in elevated CRP expression and secretion. ROS analysis of the samples showed that a minimal amount of ROS are produced by HepG2 cells in response to these concentrations of arsenic. In addition, treatment of FvB mice with 100 ppb sodium arsenite in the drinking water for six months starting at weaning age resulted in dramatically higher levels of CRP in both the liver and inner medullary region of the kidney. Further, mouse Inner Medullary Collecting Duct cells (mIMCD-4), a mouse kidney cell line, were stimulated with 10 ng/ml CRP which resulted in activation of NFκB. Pretreatment with 10 nM Y27632, a known Rho-kinase inhibitor, prior to CRP exposure attenuated NFκB activation. These data suggest that arsenic causes the expression and secretion of CRP and that CRP activates NFκB through activation of the Rho-kinase pathway, thereby providing a novel pathway by which arsenic can contribute to metabolic syndrome and cardiovascular disease.

Introduction

The Centers for Disease Control (CDC) estimates that 34% of U.S. adults meet the criteria for metabolic syndrome which includes atherogenic dyslipidemia, elevated blood pressure, insulin resistance (with or without glucose intolerance), a proinflammatory state and or a prothrombotic state. All of these factors, in addition to elevated body mass index, contribute to the risk of developing cardiovascular disease and type II diabetes (Fauci, 2008; Lara-Castro *et al.*, 2007). An individual's risk for developing metabolic syndrome is multifaceted and

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Conflict of Interest Statement

The authors declare that there are no conflicts of interest.

includes a sedentary lifestyle, as well as, genetic and environmental factors. One of these environmental factors is arsenic exposure.

Arsenic is a naturally occurring metalloid that has contaminated ground water around the world including Western, Midwestern and Northeastern areas of the United States, largely as a result of minerals dissolving from weathered rocks and soils. The most common route of human exposure to arsenic is due to ingestion of contaminated ground water. Arsenic has been recognized as a human toxicant since ancient times and chronic arsenic exposure contributes to the development of cancers of the skin, kidney, lung, bladder, and liver (Achanzar *et al.*, 2002; Berg *et al.*, 1997; Bredfeldt *et al.*, 2006; Chappell *et al.*, 1999; Chen *et al.*, 1988). Additionally, a variety of non-cancerous conditions such as neurological effects, and metabolic syndrome associated disease states such as diabetes mellitus, hypertension and atherosclerosis have been associated with arsenic exposure (Coronado-Gonzalez *et al.*, 2007; Lai *et al.*, 1994; Lemaire *et al.*, 2011; Martinez-Finley *et al.*, 2009; Navas-Acien *et al.*, 2008; Navas-Acien *et al.*, 2009; States *et al.*, 2009; Tseng *et al.*, 2000; Wang *et al.*, 2002; Xue *et al.*, 2011).

More recently studies involving exposure to low doses ($<2 \mu\text{M}$) of arsenite have gained attention as researchers have observed that these low arsenite exposures can lead to toxicities more closely related to metabolic syndrome than to cancer (Lemaire *et al.*, 2011; Xue *et al.*, 2011). For example, Xue *et al.* showed that exposure to arsenite as low as $0.25 \mu\text{M}$ decreased phosphorylated AKT levels and ultimately led to a decrease in glucose uptake and insulin resistance in the 3T3-L1 adipocytes. Similarly, Lemaire *et al.* recently showed that ApoE $^{-/-}$ mice exposed to arsenite levels as low as 200 ppb had more atherosclerotic plaques than mice exposed to higher arsenite concentrations (1000 ppb). In addition a recent study published by Sanchez-Soria *et al.*, FvB mice were exposed to 100 ppb arsenite via drinking water and were found to be hypertensive. (Sanchez-Soria 2012).

Inflammation has long been associated with the formation of atherosclerosis and the development of insulin resistance. Interleukin-6 (IL-6) is one of many pro-inflammatory cytokines that are secreted under acute inflammatory conditions. IL-6 has been shown to induce C-reactive protein (CRP) expression (Pepys *et al.*, 2003). CRP is a member of the pentraxin calcium dependent family of proteins. CRP is an acute phase protein found in serum and its levels rise in response to inflammation (Thompson *et al.*, 1999). One of the physiological roles of CRP is to activate the complement system, a part of the innate immune system that is activated by the adaptive immune system in response to pathogens, via the C1q complex (Travers *et al.*, 2001). The C1q complex is composed of peptides that together function as an attachment to the fixing sites in immune complexed immunoglobulin. CRP is synthesized by the liver in response to inflammatory factors, such as IL-6, released by macrophages and adipocytes (Thompson *et al.*, 1999). Elevated serum levels of CRP are amongst the more reliable and accessible clinical indicators of inflammation and is a predictive tool for increased coronary heart disease and type II diabetes risk and glucose disorders (D'Alessandris *et al.*, 2007; Festa *et al.*, 2000; Festa *et al.*, 2002; Ford, 1999; Pradhan *et al.*, 2001; Temelkova-Kurktschiev *et al.*, 2002). Multiple studies have shown that CRP is associated with impaired glucose tolerance, impaired fasting glucose, type II diabetes, insulin resistance and with the formation and development of atherosclerosis. Furthermore, CRP is not just a biomarker for these disease states, but also an active player in their development. In a study reported by D'Alessandris *et al.* treatment of L6 skeletal muscle cells with 10 ng/mL of CRP, levels equivalent to those found in diabetic patients, resulted in increased phosphorylation of insulin receptor substrate-1 (IRS-1) and insulin receptor substrate-2 (IRS-2) at serines 307 and 612, respectively. Phosphorylation of IRS at these sites results in the deactivation of insulin signaling, a decrease in glucose

transporter (GLUT4) translocation to the plasma membrane and decreased glucose uptake (D'Alessandris *et al.*, 2007).

Chronic low-level exposure to arsenite has been shown to elicit a pro-inflammatory response (Ahmed *et al.*, 2011; Banerjee *et al.*, 2011; Liu *et al.*, 2001; Wu *et al.*, 2003). Here we report that prolonged low level exposure to arsenite (< 2 μ M) results in increased CRP expression and secretion in the hepatic cell line, HepG2. We show that arsenite induction of CRP leads to activation of NF κ B which could further contribute to inflammation and inflammatory disease states such as atherosclerosis and insulin resistance. Further, we demonstrate that low level exposure (100 ppb) to arsenite can also induce CRP expression in FvB mouse liver and kidney. Although, historically mouse CRP has not been viewed as an important indicator of disease state, our data show that CRP may be a much more predictive indicator of arsenite exposure across species and a key molecule in elucidating low dose arsenic-related toxicities.

Methods

Reagents

LDH assay & CRP ELISA kits were purchased from Cayman Chemical (Ann Arbor, MI); anti-CRP and anti-GAPDH antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA); anti-actin antibody was purchased from Affinity Bioreagents (Golden, CO). Sodium arsenite (dissolved in MilliQ H₂O) was purchased from Sigma Aldrich (St. Louis, MO). Recombinant human CRP and anti-CRP antibody (immunohistochemistry) were purchased from R&D systems (Minneapolis, MN). 2',7' dichlorofluorescein diacetate (DCFH-DA) dye was purchased from Invitrogen (Carlsbad, CA). Mouse on Mouse Biotinylation Kit and immunohistochemistry reagents were obtained from Biocare Medical (Concord, CA). Rho-Inhibitor Y27632 was obtained from Calbiochem (EMD Chemicals, Gibbstown, NJ). Dual-Luciferase Reporter Assay system® was obtained from Promega (Madison, WI).

Mice

FvB female mice were purchased from Harlan (Harlan Laboratories Inc, WI, USA). FvB mice are a mouse strain inbred for the Fv1b allele which confers sensitivity to the Friend leukemia virus B strain. Mice were housed in sterile microisolator cages and provided diet (2019 Teklad Global 19% Protein Extruded Rodent Diet, Harlan Laboratories Inc, WI) and water *ad libitum* with either 100 ppb of sodium arsenite (NaAsO₃, Sigma, St. Louis, MO) or 100 ppb sodium chloride, to control for sodium intake (VWR, Aurora, CO) as previously reported (Sanchez-Soria, 2011). Water was purified through reverse osmosis and water packs were replaced weekly. Mice were exposed to treatments starting at day 21 and maintained on treatment for 22 weeks. Arsenite concentration in water was verified by inductively coupled plasma mass spectrometry (ICP-MS) by the Analytical Section of the Hazard Identification Core of the Superfund Research Program at the University of Arizona. Animals were euthanized by CO₂ asphyxiation and liver and kidneys collected for the studies. In addition, serum was collected and submitted to the University Animal Care Pathology Services for creatine analysis. All animal use and experimental protocols followed University of Arizona Institutional Animal Care and Use Committee (IACUC) regulations and remained in accordance with institutional guidelines.

Cell Culture

HepG2 cells, a human hepatoma cell line, were obtained from ATCC and cultured in DMEM containing 10% FBS and 1% penicillin-streptomycin (PS) and maintained at 5% CO₂ at 37°C. Mouse Inner Medullary Collecting Duct (mIMCD-4) kidney cells were kindly

provided by Dr. Heddwen Brooks from the University of Arizona Department of Physiology. These were maintained in DMEM-F12 media containing 5% FBS and 1% PS at 5% CO₂ at 37°C.

LDH Assay

HepG2 cells were cultured to 70% confluence in DMEM medium containing 10% FBS and 1% PS in a 96 well plate. HepG2 cells were then serum starved overnight and arsenic serum free medium containing arsenic at concentrations of 0, 0.13, 0.4, 1, 2 or 3 μ M sodium arsenite was added for up to 48 hours to the appropriate wells. LDH assay was performed per the manufacturer's protocol. Absorbance at 490 nm was read on VersaMax microplate reader using Softmax Pro 4.7 software (Molecular Devices, Sunnyvale, CA) and results analyzed using GraphPad Prism 5 (GraphPad, San Diego, CA).

CRP ELISA

HepG2 cells were seeded at 5×10^5 in a 6-well plate and serum starved overnight the following day when they reached 80% confluency. Cell medium was changed to DMEM containing 1% PS and either 0, 0.13, 0.67, or 2 μ M sodium arsenite for either 24 or 48 hours. Medium was removed from each plate and analyzed for CRP secretion using an ELISA kit from Cayman Chemical using the manufacturer's protocol. Absorbance was read at 450 nm on VersaMax microplate reader using Softmax Pro 4.7 software (Molecular Devices, Sunnyvale, CA) and results analyzed using GraphPad Prism 5. (GraphPad, San Diego).

Treatment and Immunoblotting

HepG2 cells were seeded at 5×10^5 and serum starved overnight the following day when they reached 80% confluency. Cell medium was changed to DMEM containing 1% PS and either 0, 0.13, 0.67 or 2 μ M sodium arsenite for either 24, 48 or 72 hours. Cells extracts were prepared using lysis buffer containing 70 mM β -glycerol phosphate, 1 mM EGTA, 0.1 mM Na₃VO₄, 1 mM DTT, 2 mM MgCl₂, 0.5% Triton X-100 and 200 μ M PMSF, 5 μ g/ml leupeptin and 10 μ l/ml aprotinin (A-6279, SIGMA, St Louis, MO). Protein concentration was measured using BSA as a standard and the Coomassie dye-binding method of Bradford (Bradford, 1976); 200 μ g of cell lysates were resolved by SDS-PAGE. Proteins were transferred to a nitrocellulose membrane. Membrane was blocked with 5% milk in TTBS and probed with anti-CRP antibody (Santa Cruz Biotechnology; 1:1000) in 5% milk in TTBS for 1 hour at room temperature with mild agitation. Membrane was then washed three times with TTBS and then incubated with secondary anti-rabbit antibodies (1:5000) in 5% milk in TTBS for 1 hour at room temperature with mild agitation. Proteins were detected by enhanced chemiluminescence and band densities were quantified by densitometry. To normalize the blots for protein levels, blots were reprobed with the appropriate primary antibodies after being immunoblotted with anti-CRP antibodies. Frozen livers were pulverized using liquid nitrogen and lysates prepared using the above described lysis buffer. Protein concentration was measured using the Bradford method and 200 μ g of cell lysate was resolved by SDS-PAGE. CRP protein was detected using anti-CRP antibody (Santa Cruz Biotechnology).

ROS

HepG2 cells were seeded at 5×10^5 and serum starved overnight the following day when they reached 80% confluency. Next, the cells were treated with either 0.13 μ M or 0.67 μ M NaAsO₃ for various times. The cells were washed with 1 $\times$ PBS and incubated with 10 μ M DCFHDA (Invitrogen) dye at 37°C for 30 minutes. Cells were trypsinized and once cells became detached from well, trypsin was neutralized with cell medium. The cells were washed once with phosphate buffered saline (PBS) and resuspended in 300 μ l PBS to a final

cell concentration of $3.3 \times 10^6/\mu\text{l}$ Samples were analyzed by flow cytometry on LSR II Flow cytometer (BD Biosciences, Sparks, MD). Fluorescence of DCFHDA was collected through the 488 nm bandpass filter. Data were analyzed using FACS Diva software (BD Biosciences, Sparks, MD).

Immunohistochemistry

Immunohistochemical staining for CRP was performed using a mouse monoclonal antibody (R&D Systems, Minneapolis, MN) with the Mouse on Mouse Biotinylation Kit. Briefly, formalin-fixed, paraffin-embedded mouse kidney samples were de-paraffinized in xylene followed by rehydration in an ethanol gradient. Endogenous peroxidase activity was blocked by incubation in Peroxidase. Antigen retrieval was achieved by low-power microwave in citrate buffer (pH 6.0) for 6 minutes. Background IgG blocking was conducted by incubation in Background Sniper (Biocare Medical, Concord, CA) for 15 minutes at room temperature. Biotinylation of the primary antibody was performed according to the manufacturer's recommendations, assuming an IgG concentration of 250 $\mu\text{g/mL}$. Biotinylated primary antibody was applied to the samples at a concentration of 1:100 overnight at 4°C. Antibody binding was detected by conjugation with HRP solution for 15 minutes at room temperature and color development with DAB Chromogen for 2 minutes followed by quenching in ddH₂O. Samples were counterstained with CAT Hematoxylin for 1 minute. Samples were de-hydrated in an ethanol gradient followed by xylene, and coverslips were fixed with Cytoseal (Richard-Allan Scientific, Kalamazoo, MI). All samples were imaged with a Leica DM4000B microscope with a Leica DFC450 camera using Leica Application Suite v.4 acquisition software.

Transfection & Luciferase Assay

Mouse inner medullary cells (mIMCD-3) were seeded at 5×10^5 cells/well in 6-well plates. The cells were co-transfected the following day with renilla (50 ng/ μl) and NF κ B (6 $\mu\text{g}/\mu\text{l}$) pGL4 luciferase vectors (Promega) using Fugene 6. The day following transfection, cells were pretreated with 10 nM Y27632, a Rho-kinase inhibitor (Calbiochem), for 1 hr or PBS (vehicle) and then co-treated with 10 ng/mL CRP for either 12 or 24 hours. Following treatment, dual luciferase reporter assay was performed following the manufacturer's protocol and activity was measured on TD-20/20 luminometer (Turner Designs, Sunnyvale, CA).

Statistical analysis

Data are mean $\pm$ standard error (SEM). For statistical comparison, data were analyzed by One-way or Two-way ANOVA, followed by either Dunnett's multiple comparison's test or by Bonferroni's post-hoc test depending on the type of analysis. Experiments were repeated a minimum of three times. (GraphPad Prism version 5; GraphPad, San Diego, CA, USA). $p < 0.05$ was considered statistically significant.

Results

Arsenite induces CRP protein expression and secretion

In order to investigate arsenite dependent induction and secretion of CRP in the hepatoma cell line, HepG2 was used, as CRP secretion and protein expression was characterized in this cell line (Depraetere *et al.*, 1991). HepG2 cells were seeded at 2×10^4 cells per 96 well plate. The next day cells were exposed to 0, 0.13, 0.4, 1, 2, or 3 μM arsenite for 24, 48 or 72 hours and a lactate dehydrogenase (LDH) assay was performed in order to determine if arsenite is cytotoxic to HepG2 cells. The measured LDH levels for the arsenite-treated samples were well below the lowest standard measurement (625 $\mu\text{U/mL}$), indicating that

these concentrations of arsenite were not cytotoxic to the HepG2 cells (data not shown). Based on these results, HepG2 cells were exposed to 0, 0.13 and 0.67 μM arsenite for 24 or 48. We observed that arsenite induced CRP expression in a dose and time dependent manner for up to 48 hrs (Figure 1a). At 72 hours of treatment there was no observable difference between control and treated cells. We believe that this is because, as Depraetere *et al.* previously reported, HepG2 cells have the ability to express CRP as they become overconfluent and by 72 hours of treatment, the control cells had produced sufficient CRP to mask any arsenite -induced CRP expression (Depraetere *et al.*, 1991). When mRNA was measured by qPCR using CRP primers we did not observe a measurable difference between control and treated samples (data not shown) suggesting that arsenite is acting at the translational level to induce CRP protein expression. When HepG2 cells were treated with arsenite for up to 24 hours to investigate the time needed for maximal elevation of CRP protein levels, we observed a time dependent increase in CRP that remained elevated up to 24 hours of exposure and then seemed to commence a decline (Figure 2b). These data demonstrate that low doses of arsenite induce CRP synthesis in HepG2 human hepatocytes.

In order to determine if the CRP produced by HepG2 cells was being secreted from the cells, we collected a sample from each experimental culture for CRP by enzyme linked immunosorbent assay (ELISA). We observed that CRP was indeed secreted from the cells with maximum secretion occurring at 4 hours post treatment that was maintained elevated up to 24 hours over control samples (Figure 3).

Arsenite does not induce CRP expression and secretion via ROS

Others have reported that a mechanism by which arsenic induces some of its injurious effects is through production of reactive oxygen species (ROS) (Sturlan *et al.*, 2003; Zhu *et al.*, 1999). In order to determine the mechanism by which arsenite induces CRP protein expression, HepG2 cells were treated with either 0.13 or 0.67 μM arsenite for up to 8 hours and ROS was measured using DCFHA dye and flow cytometry. A minimal amount of ROS were produced in response to 0.13 μM and 0.67 μM doses of arsenite, however these did not reach statistical significance (Figure 4). These data suggest that ROS is not the mechanism by which arsenite produces an increase in CRP protein levels and secretion.

Arsenite exposure induces CRP expression in FvB mice livers

In order to determine if arsenite could have similar effects *in vivo* as it did in HepG2 cells, we obtained livers from FvB mice that had been exposed to 100 ppb arsenite via drinking water for 6 months from weaning (Sanchez-Soria, 2011). Liver lysates were prepared and proteins resolved by SDS-PAGE. We observed that the mice that had been exposed to arsenite had measurably increased levels of CRP protein than the control mice (Figure 5, lanes c through e).

CRP Detection Increases in Response to Arsenic Treatment

Serum levels of CRP are well known to correlate with increased risk of coronary heart disease. In addition, immunohistochemical staining of patient kidney biopsies demonstrates increased CRP staining in diabetic patients (Schwedler *et al.*, 2003), indicating a relationship between diabetes and CRP. To further establish a relationship between arsenic and CRP, we analyzed mouse kidney sections for CRP protein expression after chronic arsenic exposure in drinking water. We used kidney tissue from the same mice that Sanchez-Soria *et al.* reported to be hypertensive in response to 100 ppb arsenite (Sanchez-Soria, 2011). CRP staining was differentially manifested throughout kidney sections of formalin fixed, paraffin-embedded tissue as assessed by immunohistochemical staining for CRP (Figure 6a). In mice not treated with arsenic, CRP staining was minimal in the cortex surrounding tubules (open arrowhead left panel figure 6a) and increased progressively towards the

medulla (Figure 6a). This staining pattern was mirrored in arsenic-treated animals. However, the intensity of CRP staining in arsenic-treated mice was noticeably greater than that of control with the majority of positive staining centralized to the medullary region (compare open arrowhead left panel and black arrowhead right panel of figure 6a). In summation, the present data indicate that CRP expression is induced throughout the kidney in response to arsenic exposure.

CRP induces NF κ B activation

A hallmark of metabolic disease is inflammation. A pivotal event involved in inflammation is NF κ B activation (Baeuerle *et al.*, 1996; Baeuerle *et al.*, 1994). NF κ B is activated by a number of stimuli including inflammatory cytokines, ROS and Rho-kinase (Baeuerle *et al.*, 1994). In order to determine if the CRP found in the tubule cells of the FvB mouse kidneys could have signaling consequences that could contribute to metabolic disease states through activation of NF κ B, we used a NF κ B luciferase reporter assay and mouse inner medullary cells (mIMCD-3) in lieu of mouse kidney. Cells were stimulated with 10 ng/mL CRP for either 12 or 24 hours and luciferase activity was measured. CRP activated NF κ B in a time dependant manner (Figure 6b). As previously mentioned, the Rho-kinase pathway has been shown to activate NF κ B (Dong *et al.*, 2010; Montaner *et al.*, 1998). In addition, Rho-kinase has been shown to play a key role in the progression of cardiovascular disease by activating and mediating inflammatory agonists and thereby promoting the process of atherosclerosis (Baeuerle *et al.*, 1996; Nossaman *et al.*, 2010). To determine whether Rho kinase functions in CRP-dependent signaling, mIMCD-3 cells were incubated with Y27632, a Rho kinase inhibitor (Ishizaki *et al.*, 2000) prior to CRP exposure. We observed that inhibition of Rho-kinase with Y27632 attenuated NF κ B activation by CRP indicating that CRP activates NF κ B through regulation of Rho kinase (Figure 6c). These data show that CRP produced in response to low dose arsenite treatment targets the medullary region of the kidney. Although NF κ B activity was not measured in mouse kidneys after arsenite exposure, CRP-dependent activation of NF κ B was observed in mIMCD-3 cells. In summary we demonstrate CRP staining in the kidney and NF κ B activation in a kidney cell line result in the transcription of inflammatory cytokines *in vivo* that have the ability to contribute to various disease states including metabolic disease.

Discussion

Over the past decade, investigators have recognized a relationship between arsenite exposure and metabolic diseases (Chen *et al.*, 1996; Coronado-Gonzalez *et al.*, 2007; Lai *et al.*, 1994; Lemaire *et al.*, 2011; Navas-Acien *et al.*, 2008; Navas-Acien *et al.*, 2009; Sanchez-Soria, 2011; States *et al.*, 2009; Tseng *et al.*, 2000; *et al.*, 2002; Xue *et al.*, 2011). Multiple epidemiological and mechanistic studies have shown that low to moderate exposure to arsenite increases the risk for metabolic related diseases such as hypertension, insulin resistance, and cardiovascular diseases including atherosclerosis and arteriolosclerosis (Lemaire *et al.*, 2011; Xue *et al.*, 2011). It has been observed that in many cases, lower levels of arsenite exposure produce more aberrant effects than higher doses of arsenite. For example, Lemarie *et al.* showed that ApoE $^{-/-}$ mice that were treated with either 200 ppb or 1000 ppb NaAsO₃ had atherosclerotic lesions. However, the ApoE $^{-/-}$ mice that were treated with 100 ppb NaAsO₃ displayed more atherosclerotic plaques than the mice treated with 1000 ppb. Likewise, here we show that low dose (0.13 μ M to 2 μ M) exposure to arsenite causes the protein expression and secretion of CRP in a dose and time dependent manner, with the lower doses (0.13 and 0.67 μ M) producing greater CRP protein expression than the higher dose (2 μ M) (Figure 2).

It has been established that patients with elevated levels of serum CRP have an increased risk of developing a multitude of metabolic disease-related malignancies including impaired

glucose tolerance, impaired fasting glucose, type II diabetes, insulin resistance and the formation and development of atherosclerosis (Festa *et al.*, 2000; Festa *et al.*, 2002; Ford, 1999; Pradhan *et al.*, 2001; Temelkova-Kurktschiev *et al.*, 2002). A recent study performed by D'Alessandris *et al.* showed that CRP is more than a clinical marker to indicate inflammation or increased risk for disease development, CRP is also an active contributor to disease state (D'Alessandris *et al.*, 2007). In their study, D'Alessandris treated L6 myocytes, a skeletal muscle cell line that is insulin responsive, with 10 ng/ml hCRP, levels equivalent to those found in diabetic patients. They found that CRP inhibited insulin signaling via the phosphorylation of serines 307 and 612 on the insulin receptor substrates, IRS-1 and IRS-2, respectively. They also found that 10 ng/ml CRP was sufficient to inhibit the translocation of the glucose transporter, GLUT4, to the cell membrane. Additionally, increased CRP serum levels are associated with atherosclerotic plaque formation and cardiovascular disease (Pepys *et al.*, 2006). Therefore it stands to reason that if low dose arsenite exposure can induce CRP expression and secretion to rise, then serum CRP could be a contributor to the insulin resistance, hyperglycemia, hyperinsulinemia and atherosclerotic plaque formation and hypertension observed in association with inorganic arsenic exposure (Chen *et al.*, 1996; Coronado-Gonzalez *et al.*, 2007; Lai *et al.*, 1994; Lemaire *et al.*, 2011; Martinez-Finley *et al.*, 2009; Navas-Acien *et al.*, 2008; Navas-Acien *et al.*, 2009; Sanchez-Soria, 2011; States *et al.*, 2009; Tseng *et al.*, 2000). In order to explore this hypothesis further we obtained liver and kidney samples from a study performed by our collaborators in the Camenisch research laboratory on mice that had been chronically exposed to 100 ppb arsenite and had developed hypertension as a result. Here we show that low dose arsenite induced CRP protein expression both *in vitro* (HepG2) and *in vivo* (figures 2a & b and 5). In addition elevated CRP secretion was detected in HepG2 cells suggesting that arsenite could contribute to elevated serum CRP levels *in vivo* and contribute to overall malignancies associated with increased serum CRP as discussed above.

Others have reported ROS production in response to arsenic exposure (Druwe *et al.*, 2010; Sturlan *et al.*, 2003; Suzuki *et al.*, 2009; Zhu *et al.*, 1999) as a mechanism by which arsenic mediates its toxic effects. In order to determine if the mechanism of CRP induction is through production of ROS we performed flow cytometry analysis using DCFHA dye. We found that a minimal amount of ROS were produced in response to low dose arsenite exposure but that the ROS production did not correlate with CRP expression and secretion (compare figures 1b, 2 & 3 at the 1 through 8 hr timepoints) indicating that CRP expression and secretion were not through the production of ROS by arsenite exposure.

In order to establish if elevated CRP levels observed in both our *in vivo* and *in vitro* models coincided with increased CRP staining observed in diabetic patients, the kidneys of the FvB mice underwent immunohistochemical staining for CRP. In control tissues, CRP detection was minimal in the cortex and increased progressively towards the medulla. This detection pattern was similar in arsenic-treated animals. The detection of CRP in arsenic-treated rodents was noticeably greater than that of non-treated mice with the majority of positive staining centralized to the medullary region. The data indicate that CRP protein expression is induced throughout the kidney in response to arsenic exposure (Figure 6a), indicating that the kidney is a target organ for arsenic exposure.

From experiments performed by D'Alessandris *et al.*, we hypothesize that the increased presence of CRP in the mouse kidneys is not inert and plays a role in the associated inflammation observed in insulin related diseases, cardiovascular disease and metabolic syndrome (D'Alessandris *et al.*, 2007). In order to test our hypothesis we seeded mouse inner medullary cells (mIMCD-3) and cotransfected the cells with renilla and NF κ B luciferase vectors. We then treated the cells with (10 ng/ml) CRP levels equivalent to those found patients at risk for developing metabolic syndrome (D'Alessandris *et al.*, 2007), and we

observed that CRP had the ability to activate NF κ B in a time dependant manner (Figure 6b). It was reported that FvB mice exposed to 100 ppb arsenite via drinking water became hypertensive as a result of exposure (Sanchez-Soria, 2011). Rho-kinase has been shown to play a role in a number of cardiovascular disease including atherosclerosis. Activation of NF κ B by Rho-kinase leads to the transcription of various molecules that have been shown to contribute to inflammation, metabolic syndrome and cardiovascular disease including plasminogen activator inhibitor 1, PAI-1, nitric oxide (NO), and IL-6 (Binder *et al.*, 2002; Calo *et al.*, 2006). We therefore hypothesized that CRP activated NF κ B through the Rho-kinase pathway. In order to test our hypothesis we employed the use of the mIMCD-3 cell culture model as the detection of CRP in the kidney was greatly increased in the medulla in arsenite exposed FvB mice. We observed that CRP was unable to activate NF κ B in the presence of the Rho kinase inhibitor Y27632, implicating a role for the Rho kinase pathway in the activation of NF κ B. These data show that CRP produced due to low dose arsenite treatment has the ability to affect NF κ B activity. NF κ B activation may then result in the transcription of inflammatory cytokines that could contribute to various disease states including metabolic syndrome. Others have observed that arsenite activates NF κ B (Barchowsky *et al.*, 1996; Escudero-Lourdes *et al.*, 2010; Fry *et al.*, 2007; Ghosh *et al.*, 2009) but the mechanism of by which arsenic activates NF κ B is still unknown. Our data show that arsenite causes the protein expression and secretion of CRP which then activates the Rho-kinase pathway and results in NF κ B activation.

In mice, CRP is typically considered to be a trace protein whose concentrations increase only modestly in an acute-phase response and a clear physiological role for mouse CRP has yet to be elucidated (Pepys *et al.*, 2003). However, 100 ppb sodium arsenite exposure has been shown to trigger hypertension (Sanchez-Soria, 2011). Upon immunohistochemical analysis of kidneys originating from the mice in the study by Sanchez-Soria *et al.* we found that in control tissues, CRP staining was minimal in the cortex and increased progressively towards the medulla. The intensity of CRP staining in arsenic-treated rodents was noticeably greater than that of control with the majority of positive staining centralized to the medullary region. The present data indicate that CRP protein expression is induced throughout the kidney in response to arsenic exposure (Figure 6a). Furthermore, western blot analysis probing for CRP of liver lysates originating from the same study found that the arsenic treatment caused a remarkable increase in CRP protein in liver lysates as compared to control samples (Figure 5). These data collectively show that arsenite causes an elevation in CRP protein levels and secretion *in vivo* and *in vitro*. We further show that CRP is an active molecule that activates NF κ B transcription through Rho kinase activation and this is a plausible mechanism for the hypertension observed by Sanchez-Soria *et al.* in response to sodium arsenite treatment. Significantly, these data show that in addition to being a clinical diagnostic tool for cardiovascular and diabetic risk, CRP may also be a clinical indicator for arsenite exposure and toxicological risk.

A major comorbidity of metabolic syndrome is cardiovascular disease, which continues to carry the highest mortality rates of any disease in the United States each year. In addition, a large percentage of patients suffering from diabetes also suffer from cardiovascular complications placing these individuals at a two to four-fold higher risk of mortality due to cardiovascular disease than non-diabetics (Kvan *et al.*, 2007; Norhammar *et al.*, 2004). Our data show that exposure to environmentally relevant concentrations of arsenite can contribute to metabolic syndrome by increasing the serum levels of CRP. Multiple studies have shown that patients with elevated levels of CRP have increased risk of developing diabetes (Festa *et al.*, 2000; Festa *et al.*, 2002; Ford, 1999; Pradhan *et al.*, 2001; Temelkova-Kurktschiev *et al.*, 2002), hypertension and cardiovascular disease (Pepys *et al.*, 2003; Pepys *et al.*, 2006). CRP has also been shown to exacerbate ischemic necrosis in a complement-dependent manner. Moreover, *in vivo* studies have shown that inhibition of CRP may be a

potential therapeutic target for myocardial and cerebral infarcts (Pepys *et al.*, 2006). Our data demonstrate that the environmental contaminant, arsenic, increases CRP production implicating arsenic as a contributor to metabolic disease.

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Highlights

- Exposure to arsenic can induce the expression & secretion of CRP.
- Mice treated with NaAsO₃ showed higher levels of CRP in both the liver & kidney.
- mIMCD-3 were stimulated with CRP which resulted in activation of NFκB.
- CRP activates NFκB through activation of the Rho-kinase pathway.
- Data provide novel pathway for arsenic role in metabolic & cardiovascular disease.

LDH Concentration in Response to NaAsO₃

Figure 1.

LDH Assay. HepG2 cells were cultured to 70% confluence in DMEM medium containing 10% FBS and 1% PS in a 96 well plate. HepG2 cells were then serum starved overnight and serum free medium containing arsenic at concentrations of 0, 0.13, 0.4, 1, 2 or 3 μM sodium arsenite was added for up to 48 hours to the appropriate wells. LDH assay was performed per the manufacturer's protocol. Absorbance at 490 nm was read on a VersaMax microplate reader using Softmax Pro 4.7 software (Molecular Devices, Sunnyvale, CA) and results analyzed using GraphPad Prism 5 (GraphPad, San Diego, CA). Although LDH levels increased overtime these levels were not caused by arsenite exposure and were still well below the lowest positive LDH standard (625 μU/mL). Bars represent relative fluorescent units normalized against control (OD 490 nm) for three independent experiments. Samples were analyzed using One-Way ANOVA and using Dunnet's post-hoc test.

A

B

Figure 2.

a: HepG2 cells were seeded at 5×10^5 cells/well in 6-well plates. The next day cells when cells reached 70% confluence, the cells were serum starved overnight and then DMEM 1% PS containing 0, 0.13, 0.67 or 2 μ M NaAsO₃ (sodium arsenite) was added for either 24, 48 or 72 hours (72 hr data not shown). We observed that CRP protein expression was elevated in response to sodium arsenite exposure after 24 and 48 hours of treatment. The graph represents mean densitometry $\pm$ standard deviation from three independent experiments. * $p < 0.05$

b: HepG2 cells were seeded at 5×10^5 cells/well in 6-well plates. The next day cells when cells reached 70% confluence, the cells were serum starved overnight and then DMEM 1% PS containing 0.67 μ M NaAsO₃ (arsenite) was added for either 0, 15 min, 30 min, 1 hr, 4 hr, 8 hr or 24 hr. We observed that elevated CRP protein levels commenced to be elevated after just 15 minutes of exposure to arsenite and reached maximum expression at 8 hours and remained elevated through the 24 hr timepoint.

CRP SECRETION IN RESPONSE TO ARSENIC

Figure 3.

HepG2 cells were seeded at 5×10^5 cells/well in 6-well plates. The next day cells when cells reached 70% confluence, the cells were serum starved overnight and then DMEM 1% PS containing either 0, 0.13 or 0.67 μM NaAsO₃ (arsenite) was added for either 0, 15 min, 30 min, 1 hr, 4 hr, 8 hr or 24 hr. A media sample from each plate was removed from each plate and analyzed for CRP secretion via ELISA. Absorbance at 490 nm was read on plate reader. The graph represents CRP fold over basal levels found in control (no arsenite treatment) $\pm$ SEM for three independent experiments; samples were analyzed using One-Way ANOVA and Bonferroni post-hoc test. * $p < 0.05$.

ROS in HepG2 in response to arsenite

Figure 4.

HepG2 cells were seeded at 5×10^5 cells/well in 6-well plates. The next day cells when cells reached 70% confluence, the cells were serum starved overnight and then DMEM 1% PS containing 0.13 or 0.67 μM NaAsO₃ was added for indicated times. Then cells were washed with PBS and 10 μM DCFHA dye was added to each well at 37°C for 30 minutes. Cells were trypsinized and once cells became detached from well, trypsin was neutralized with cell medium. The cells were washed once with phosphate buffered saline (PBS) and resuspended in 300 μl PBS to a final cell concentration of 1×10^6 . Samples were analyzed by flow cytometry on LSR II Flow cytometer (BD Biosciences, Sparks, MD). Fluorescence of DCFHDA was collected through the 488 nm bandpass filter. Data were analyzed using FACS Diva software (BD Biosciences, Sparks, MD). Although some ROS were produced at the 0.13 μM dose within the first hour of arsenite treatment, the ROS production was not statistically significant over control. Bars represent relative fluorescence units normalized against control $\pm$ SEM for three independent experiments. Samples were analyzed using Two-Way ANOVA and Dunnett's post-hoc test t.

Figure 5.

FvB female mice were exposed to either 100 ppb of sodium arsenite or 100 ppb sodium chloride as previously reported (Sanchez-Soria, 2011). Water was purified through reverse osmosis and water packs were replaced weekly. Mice were exposed to treatments starting at day 21, and maintained on treatment for 22 weeks. Animals were euthanized by CO₂ asphyxiation, livers were excised and snap frozen in liquid nitrogen. Samples were prepared by pulverizing liver samples using liquid nitrogen and lysates prepared using lysis buffer with 200 µg of each cell lysate resolved by SDS-PAGE.

a

NF κ B Activity in Response to CRP

b

NF κ B Activity in Response to CRP

c

Figure 6.

(a) Formalin-fixed, paraffin-embedded mouse kidney from FvB mice underwent immunohistochemical staining. In mice not treated with arsenic (Left panel), CRP staining was minimal in the cortex and increased progressively towards the medulla. This staining pattern was mirrored in arsenic-treated animals (Right panel). However, the intensity of CRP staining in arsenic-treated mice was noticeably greater than that of control with the majority of positive staining centralized to the medullary region. Creatine levels were measured and found to be within normal range (0.6–1.2 mg/dL) for all samples (data not shown). (b) Mouse inner medullary cells (mIMCD-3) were seeded at 5×10^5 cells per well in 6-well plates. The cells were co-transfected the following day with renilla (50 ng/ μ l) and NF κ B (6 μ g/ μ l) pGL4 luciferase vectors (Promega) using Eugene 6. The day following transfection, cells were treated with 10 ng/mL CRP for either 12 or 24 hours. 10 ng/mL TNF α was used as a positive control. Following treatment, dual luciferase reporter assay was performed following manufacturer's protocol and activity was measured on TD-20/20 luminometer (Turner Designs, Sunnyvale, CA). (c) In order to determine if NF κ B activation was due to Rho-kinase activation, the day after transfection cells were pretreated with 10 nM Y27632, a Rho-kinase inhibitor (Calbiochem), for 1 hr or PBS (vehicle) and then co-treated with 10 ng/mL CRP for either 12 or 24 hours. Following treatment, dual luciferase reporter assay was performed following manufacturer's protocol and activity was measured on TD-20/20 luminometer. Bars represent luciferase activity normalized against control (no treatment) $\pm$ SEM for three independent experiments. Samples were analyzed using One-Way ANOVA and using Dunnet's post-hoc test. * $p < 0.05$; # $p > 0.05$.

Figure 7. Health consequences caused by elevated CRP in response to arsenite exposure in drinking water.