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In vitro particulate matter exposure causes direct and lung-mediated indirect effects on cardiomyocyte function

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Gorr MW, Youtz DJ, Eichenseer CM, Smith KE, Nelin TD, Cormet-Boyaka E, Wold LE. In vitro particulate matter exposure causes direct and lung-mediated indirect effects on cardiomyocyte function. Am J Physiol Heart Circ Physiol 309: H53–H62, 2015. First published May 4, 2015; doi:10.1152/ajpheart.00162.2015.—Particulate matter (PM) exposure induces a pathological response from both the lungs and the cardiovascular system. PM is capable of both manifestation into the lung epithelium and entrance into the bloodstream. Therefore, PM has the capacity for both direct and lung-mediated indirect effects on the heart. In the present studies, we exposed isolated rat cardiomyocytes to ultrafine particulate matter (diesel exhaust particles, DEP) and examined their contractile function and calcium handling ability. In another set of experiments, lung epithelial cells (16HBE14o- or Calu-3) were cultured on permeable supports that allowed access to both the basal (serosal) and apical (mucosal) media; the basal media was used to culture cardiomyocytes to model the indirect, lung-mediated effects of PM on the heart. Both the direct and indirect treatments caused a reduction in contractility as evidenced by reduced percent sarcomere shortening and reduced calcium handling ability measured in field-stimulated cardiomyocytes. Treatment of cardiomyocytes with various anti-oxidants before culture with DEP was able to partially prevent the contractile dysfunction. The basal media from lung epithelial cells treated with PM contained several inflammatory cytokines, and we found that monocyte chemotactic protein-1 was a key trigger for cardiomyocyte dysfunction. These results indicate the presence of both direct and indirect treatments caused a reduction in contractility as evidenced by reduced percent sarcomere shortening and reduced calcium handling ability measured in field-stimulated cardiomyocytes.

This study was designed to examine the mechanisms in which PM affects cardiovascular function by measuring the contractility and calcium handling ability of cardiomyocytes under conditions modeling the direct and lung-mediated indirect exposure of the heart to PM. To examine the direct effects of PM on cardiomyocyte function in vitro, future work will focus on elucidating the mechanisms involved in these separate pathways using in vivo models of air pollution exposure.

Air pollution; particulate matter; cardiovascular toxicology; in vitro toxicology

DETRIMENTAL EFFECTS ELICITED by the particulate matter (PM) component of air pollution on the cardiovascular system are becoming increasingly clear (52). This is most remarkably shown from retrospective cohort studies indicating the risk of cardiovascular-related death increases with various estimates ranging from 12% to 76% for every 10 μg/m³ increase in the ambient PM (23, 25–27). Increased PM exposure over periods from 1 to 30 days is also linked to subclinical findings such as increased blood pressure and ventricular mass (1, 48a). Clinical and controlled animal exposure studies suggest that systemic inflammation and oxidative stress contribute to PM-induced cardiovascular dysfunction, since increased circulatory markers of inflammation including IL-6, C-reactive protein, TNF-α, IL-1β (7, 40, 43), and markers of oxidative stress such as increased homocysteine levels and 8-hydroxy-2′-deoxyguanosine DNA adducts (2, 16, 38, 42) have been found. However, the mechanisms leading to cardiovascular dysfunction from air pollution exposure require further study.

One hypothesis is that PM is able to affect the heart directly by crossing into the bloodstream (14, 24, 28, 32, 36, 39) and indirectly by downstream consequences created by its manifestation into lung alveoli (31, 44). Understanding these processes at the cellular and molecular levels brings further insight into how the heart is affected by PM, and can provide strategies for preventing further damage when air pollution is combined with pathological processes that share a similar mechanism, such as diabetes, hypertension, and obesity.

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MATERIALS AND METHODS

Cardiomyocyte isolation and functional measurement. All animal studies were approved by the Institutional Animal Care and Use Committee at the Ohio State University. Cardiomyocytes were isolated from male Sprague Dawley rats (2 to 4 mo old) as previously described (29, 51, 52). Briefly, hearts were removed and the coronary arteries were perfused through the aorta with Liberase and trypsin until the tissue was digested. Cells were then plated on laminin-coated glass-bottom inserts (Cell MicroControls, Norfolk, VA) for functional analyses. Glass-bottom inserts were perfused with warm contractile buffer (52) within a flow chamber attached to an Olympus IX-71 microscope. Cells were stimulated (1 Hz, 3-sms duration) with a Myopac Field-Stimulator system (IonOptix, Milton, MA) at 37°C and functional properties of the cells were evaluated with the Sarcen...
Sarcomere Length Acquisition Module using the Myocam-S Digital charge-coupled device camera video imaging system (IonOptix). Analyses of 8–12 cells from 3 to 4 rats for each treatment provided the parameters of contractility (sarcomere percent peak shortening, normalized to baseline length [%PS]), systolic function (sarcomere departure velocity [+dL/dT] and time-to-90% peak shortening [TPS 90]), and diastolic function (sarcomere return velocity [−dL/dT] and time-to-90% peak relaxation [TR 90]).

**Measurement of intracellular Ca$^{2+}$ transient.** Cardiomyocytes in glass-bottom dishes were loaded with Fura-2-AM (0.5 μM) for 20 min at 25°C and then washed and treated with normal culture media for 20 min at 25°C. Fluorescence was recorded in stimulated cardiomyocytes at 37°C using a dual-excitation, single-emission system (IonOptix). Transients were analyzed for values of calcium release ($\Delta$340/380) and reuptake ($\gamma$).

**Treatment of lung epithelial cells.** Calu-3 and lung epithelial cells were obtained from ATCC and 16HBE14o- were a gift from Dr. Dieter Gruenert (University of California-San Francisco) and were cultured on permeable supports (Transwell, Corning). Calu-3 cells were derived from an adenocarcinoma whereas 16HBE cells were derived from normal bronchial epithelium. DEP (National Institute of Standards and Technology, 1650b) were prepared in culture media at a concentration of 1 mg/ml, and sonicated in 1-s bursts for 30 s using Sonic Dismembrator (Fisher Scientific) at 100% power. DEP was added in culture at various concentrations to the apical chamber of the polarized lung epithelial cells. Media from the basal chamber was collected after 24 h of treatment and frozen at −80°C until use for the treatment of cardiomyocytes. Electrical resistance of the cells was measured after treatment to ensure epithelial barrier integrity. Additionally, paracellular permeability was measured using FITC-dextran (4 kDa) added to the apical chamber. Basal media (100 μl) was collected at various times, and FITC-dextran present in the samples was measured using a fluorescence microplate reader (Victor X3, Perkin Elmer).

**Treatment of cardiomyocytes.** Cardiomyocytes were treated for 1 h with DEP; diluted to 0.25, 0.50, 1.0, and 25 μg/ml; and filtered through 5 μm filter paper to remove aggregates. A subset of cells was pretreated with a combination of N-acetyl-l-cysteine (NAC; 10 mM) and Tiron (10 mM), apocynin (10 mM), mito-tempol (10 mM), or oxypurinol (15 mM) for 1 h before DEP treatment at 25 μg/ml. Some cells were also treated with isoproterenol (ISO; 1 nm) for 3 min before measurement. In another subset of experiments, cardiomyocytes were cultured for 3 h with media from Calu-3 or 16HBE14o- conditioned cell culture (see above), which was diluted with an equivalent volume of unconditioned culture media.

**Cytokine assay.** Conditioned media from the basal and apical chambers was analyzed for levels of 25 different cytokines using the Bio-Plex Cytokine Assay (Bio-Rad Laboratories, Hercules, CA).

**Statistical analyses.** Data were assessed using Prism 6.0 (Graphpad Software, San Diego, CA) and differences were considered statistically significant when $P < 0.05$ via two-tailed Student’s $t$-test, one-way ANOVA, or two-way ANOVA with Tukey’s post hoc test where appropriate.

**RESULTS**

**DEP alters cardiomyocyte contractility.** Cardiomyocytes were treated with DEP at multiple concentrations for 1 h, and contractility was measured by assessing changes in sarcomere length at 1 Hz stimulation (Fig. 1). Cardiomyocytes treated with DEP had reduced contractility as evidenced by a signifi-
cant reduction in %PS, −dL/dT, and +dL/dt, at concentrations as low as 1 µg/ml (Fig. 1). TPS 90 was also altered at 25 µg/ml (Fig. 2), whereas TR 90 was not significantly altered.

Anti-oxidant pretreatment prevents cardiomyocyte dysfunction caused by DEP. Cardiomyocytes were treated for 1 h with anti-oxidants before DEP treatment to examine the role that reactive oxygen species (ROS) play in DEP-mediated cardiomyocyte dysfunction (Fig. 2). Pretreatment with Tiron and NAC, apocynin, or oxypurinol led to contractile characteristics similar to control cells. These results indicate that ROS from several cellular sources are partially responsible for DEP-mediated cardiomyocyte dysfunction as previously reported (55).

DEP treatment alters the contractile response to β-adrenergic stimulation. Additional cardiomyocytes were treated with 1 nM ISO to examine the effect of β-adrenergic stimulation on contractile function. DEP-treated cardiomyocytes maintained a reduction in contractility following ISO treatment compared with cells treated with ISO alone as shown by reduced %PS and −dL/dT, but there was no significant difference in +dL/dT (Fig. 3). This showed that DEP caused a diminished response to β-adrenergic stimulation in cardiomyocytes.

DEP treatment alters calcium handling of cardiomyocytes. Calcium-mediated fluorescence was measured using the calcium indicator Fura-2 in stimulated cardiomyocytes after cells were treated with DEP as indicated above. There was no significant change in Δ340/380 or Δ340/380 compared with control (Fig. 4). When cells were also treated with ISO, DEP treatment caused a significant reduction in Δ340/380 compared with control cells treated with ISO alone. Untreated and DEP-treated cells had a significant decrease in τ with ISO treatment, but Δ340/380 was only significantly increased in control cells. This indicated that β-adrenergic alterations in calcium release from the sarcoplasmic reticulum were reduced in DEP-treated cardiomyocytes.

Conditioned media from DEP-treated lung epithelial cells causes cardiomyocyte dysfunction. Calu-3 lung epithelial cells were treated with various concentrations of DEP in the apical compartment of a membrane culture system for 24 h. Isolated cardiomyocytes were then treated with media collected from the basal compartment of the culture system for 3 h, and contractility and calcium transients were measured. This conditioned media caused a reduction in %PS, −dL/dT, and +dL/dT at concentrations as low as 15 µg/cm² (or 50 µg/ml), but caused no statistically significant changes in TR 90 or TPS 90 (Fig. 5, A–E), although TR 90 trended toward a dose-dependent increase over time. Identical results were found when similar studies were performed with 16HBE14o- epithelial cells. Resistance was unchanged with particle treatment (data not shown), and epithelial barrier function of Calu-3 cells was not altered by DEP as observed in Fig. 5F. Treatment of cardiomyocytes with conditioned media from Calu-3 cells also caused a small change in calcium handling as shown by
decreases in $\Delta 340/380$, but this was not statistically significant (Fig. 6).

Polarized lung epithelial cells secrete cytokines in the serosal compartment in response to DEP. Conditioned media from Calu-3 cells was examined for secreted cytokines using the Bioplex kit for cells treated with control or 15 g/cm$^2$ DEP. Cytokines in the basal/serosal compartment that were significantly increased following DEP treatment when compared with untreated cells included IL-6, IL-8, IL-15, interferon $\gamma$-induced protein (IP)-10, monocyte chemotactic protein (MCP)-1 and regulated on activation, normal T cell expressed and secreted (RANTES) protein (Fig. 7A). All other cytokines were not altered with DEP treatment (data not shown).

Recombinant cytokines cause cardiomyocyte dysfunction. To examine the role of specific cytokines in DEP-induced cardiomyocyte dysfunction observed above, cardiomyocytes were cultured using cytokines at 50% of the concentration obtained in the bioplex assay (as the conditioned media was diluted 2:1 in normal media) and contractile function was assessed. Out of the six cytokines assessed, only MCP-1

Fig. 3. DEP treatment of cardiomyocytes causes alteration in $\beta$-adrenergic stimulation. Cardiomyocytes were treated with DEP (25 g/ml, DEP + isoproterenol (ISO)) compared with cells treated with ISO alone (control + ISO). All values were analyzed from 8 to 10 cells from 3 to 4 rats each. A: %PS. B: +dL/dT. C: −dL/dT. D: TR 90. E: TPS 90. *Significant difference compared with control + ISO, $P < 0.05$ by t-test.

Fig. 4. DEP treatment of cardiomyocytes causes altered calcium handling in response to $\beta$-adrenergic stimulation. Cardiomyocytes were treated with DEP and ISO, and their calcium-handling ability was measured compared with control and ISO treatment. All values were analyzed from 8 to 10 cells from 3 to 4 rats each. A: FURA fluorescence $\Delta 340/380$. B: calcium reuptake, $\tau$. C: representative calcium transients. *Significantly different compared with control (C); #significant difference compared with control + ISO; Significantly different compared with DEP $P < 0.05$ by 2-way ANOVA.

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reduced the %PS, +dL/dT, and −dL/dT (Fig. 7, B–F). Because MCP-1 had the only notable effect on cardiomyocyte contractility, we pretreated cardiomyocytes with a CCR2 (MCP-1 receptor) antagonist (10 μM; Santa Cruz Biotech) for 30 min before treatment with conditioned media from Calu-3 cells as above. The inhibitor was successful at preventing cardiomyocyte contractile dysfunction when compared with cells treated in normal culture media (Fig. 7, G–I). This indicated that MCP-1 plays a significant role in cardiomyocyte dysfunction caused from Calu-3-mediated indirect DEP treatment.

DISCUSSION

In the current study, we investigated the effects of direct and indirect treatment of cardiomyocytes with DEP. Direct treatment of cardiomyocytes with DEP caused contractile dysfunction and alterations in calcium handling. This dysfunction was ameliorated by pretreatment with several anti-oxidants, indicating that cellular oxidative stress is an important component of the cardiovascular dysfunction in response to PM exposure. The contractile response to ISO was prevented in cardiomyo-
cytes treated with DEP, providing evidence that the cellular response to β-adrenergic stimulation is inhibited with DEP treatment. This indicates that DEP is capable of interfering with sympathetic stimulation of the heart. Furthermore, treatment of cardiomyocytes with media conditioned by DEP-treated lung epithelial cells resulted in a similar reduction in contractility and calcium handling ability. This conditioned media was found to contain increased levels of several cytokines, and treatment of cardiomyocytes with individual recombinant cytokines at the concentrations found in the conditioned media revealed that MCP-1 was the main contributor to the cardiomyocyte dysfunction observed. This was further confirmed when blocking the receptor for MCP-1 with a small molecule inhibitor before treatment with the conditioned media prevented cardiomyocyte dysfunction, suggesting that MCP-1 plays an important role in the lung-mediated indirect effects of PM on the heart.

Contractile dysfunction and reduced calcium handling that resulted from the treatment of cardiomyocytes with DEP was partially reversible with pretreatment of anti-oxidants. Interestingly, +dL/dT was unchanged compared with control at 1 μg/ml, but was significantly reduced at 0.25 μg/ml. This fact may be due to a slightly smaller standard error of the mean at the lower concentration, so it is difficult to interpret whether this is biologically relevant. We found that inhibition of oxidative stress via the ROS-scavengers NAC and tiron reduced the dysfunction present from DEP-treated cardiomyocytes. Because these are general ROS scavengers, this indicated the dysfunction observed was due to ROS, so we investigated this with more specific ROS inhibitors. Inhibition of NADPH oxidase with apocynin, inhibition of xanthine oxidase with oxypurinol, and inhibition of mitochondria-derived ROS with mito-tempo all partially restored cardiomyocyte contractility. Although we found that mitochondrial-derived ROS had less impact than the other enzymes, indicating the ROS produced from DEP-treated cells is mostly occurring from NADPH oxidase and xanthine oxidase, these data provide further targets for in vivo exposure studies. These findings support others who have found oxidative stress as a result of PM treatment of adult or neonatal cardiomyocytes and fibroblasts (18, 37, 48, 55) and support the general hypothesis that the heart is affected by PM through oxidative stress mechanisms. Due to the partial restoration of cell function found after treatment with each antioxidant, we conclude that cardiomyocytes incubated with DEP have a contractile dysfunction caused by a general state of excess ROS production from multiple cellular sources.

This study examined cytokine release in response to DEP treatment from epithelial cells cultured on a permeable support system. Because we were able to examine what was released into the basal chamber from polarized cells, we are able to use the results of our study toward the design of in vivo exposure studies. Cytokine release from epithelial cells cultured in normal culture dishes have similar increases in IL-6 and/or IL-8 when treated with DEP or other PM (4–6, 9, 11, 12, 19, 30, 41, 45, 49, 53). However, several studies found increases in TNF-α, IL-1α, and/or granulocyte macrophage colony-stimulating factor (3, 4, 9, 12, 15, 21, 45), which were not increased in our model. No studies have reported serosal secretion of MCP-1, IL-15, IP-10, or RANTES from lung epithelial cells in response to PM treatment, which provides novel direction toward determining a mechanism for cardiovascular dysfunction caused by PM.

MCP-1 has long been implicated in cardiovascular disease, normally involved in the recruitment of monocytes within the myocardium and vasculature (for a recent review, see Ref. 35). In a mouse model, MCP-1 overexpression in the heart caused heart failure, and the MCP-induced protein (MCPIP) transcription factor was increased in parallel with reductions in cardiac fractional shortening (54). Accordingly, we hypothesized that MCP-1 is released from the lungs in response to PM exposure, activating the MCP-1/CCR2 receptor causing downstream effects on cardiovascular dysfunction. Several in vivo studies have found cardiac dysfunction and increased serum cytokines after DEP exposure, in addition to alterations in the autonomic nervous system (8, 20). Here we have found that MCP-1 is capable of inducing cardiac contractile defects in vitro, implicating the importance of inflammatory cytokines in DEP-mediated contractile defects. We did not use the low level of MCP-1 found in the control conditioned media as a control against MCP-1 treatment, but rather media without MCP-1, which may slightly exaggerate the effects of MCP-1 on contractility observed. MCP-1 has been found in the serum of mice exposed long term to PM inhalation (22), and increases in other cytokines, such as IL-6, have been found in similar studies following PM exposure (13, 33). In the present study, we found that IL-6 at the concentration used did not cause contractile defects in vitro. Future studies are needed to examine the cytokine profile in controlled exposure studies and their effect on cardiac function in vivo.

Limitations of this study include the lack of particle characterization concerning the DEP in the media used to culture cardiomyocytes and lung epithelial cells. Characterization of the standardized DEP particles used in this study has been completed (34), but the size, available reactive groups, and metals present in solution in the current study are unknown, although a study with a similar sonication protocol found that the particles in solution were primarily in the range of 18 to 30 nm, placing DEP in the ultrafine (≤100 nm) range (47). The amount of DEP that translocated into the basal compartment of our system was also not measured, but we speculate this amount was relatively low, since PM has been shown to improve barrier function (46), and the amount of FITC-dextran
found in the basal compartment was similar with control or DEP treatment as shown in Fig. 5F. We used bronchial epithelial cells in this set of experiments, which may have provided a different cytokine profile than alveolar epithelial cells and/or a coculture of epithelial cells and immune cells. Nevertheless, we believe our model (Fig. 8) provides a platform for future studies examining different particle sources, chemical compositions, and sizes as well as different cell types.

Concerning the extent of particle translocation into the bloodstream and to the heart, several studies have attempted to address this using labeled particles. Studies in animals using labeled ultrafine particles have found that either less than 1% (14, 24) or a negligible amount (36) of the PM mass was found in heart tissue following exposure. In humans, studies have also found negligible lung clearance of labeled particles (28, 50), although others have found immediate notable clearance into the bloodstream after inhalation (32). Further studies are still needed that address the effects of particle charge (10), as well as duration from exposure to endpoint evaluation, since most of the studies cited above examine translocation within hours of exposure. Taking the current research into account, our in vitro concentrations likely overestimate the concentration of PM found in the heart in vivo, but nevertheless provide a platform for examining the mechanisms behind the effects of DEP and other PM types on the cardiovascular system that can be further investigated using in vivo PM exposure paradigms. Further study is also necessary to determine the pathway of PM after it translocates into the bloodstream, including the role of the liver in filtering the PM, before drawing firm conclusions on the usefulness of in vitro models such as the ones presented here.

In conclusion, we have used in vitro methodology to study both the direct and indirect pathways of PM-induced cardiomyocyte dysfunction. Directly, PM is able to induce a ROS-mediated reduction in contractility and calcium handling in cardiomyocytes, and a reduced response to β-adrenergic stimulation. Indirectly, PM induced cytokine release from lung epithelial cells that caused a similar reduction in the contractility and calcium handling of cardiomyocytes, mainly through MCP-1-mediated mechanisms. Future research will examine the relevance of these pathways using in vivo models of air pollution exposure.

REFERENCES


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