Diesel Exhaust Particles Induce Impairment of Vascular and Cardiac Homeostasis in Mice: Ameliorative Effect of Emodin

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Abstract
\textbf{Background/Aim:} There is strong epidemiological and clinical evidence that components of the cardiovascular system are adversely affected by particulate air pollutants through the generation of inflammation and oxidative stress. Emodin (1,3,8-trihydroxy-6-methylanthraquinone), which is commonly found in the roots of rhubarb plant, has strong antioxidant and anti-inflammatory effects. However, its possible protective effect on the cardiovascular effect of particulate air pollutants has never been reported before. \textbf{Methods:} We tested, in Tuck-Ordinary mice, the possible ameliorative effect of emodin on the acute (24h) cardiovascular effects of diesel exhaust particles (DEP, 1 mg/kg) or saline (control). Emodin (4 mg/kg) was administered intraperitoneally 1h before and 7h after pulmonary exposure to DEP. Twenty four h following DEP exposure, several cardiovascular endpoints were assessed. \textbf{Results:} Emodin significantly prevented the increase of leukocyte (n=8, \(P<0.001\)) and erythrocyte (n=8, \(P<0.01\)) numbers caused by DEP. Likewise, emodin abrogated DEP-induced increase of heart tissue levels of interleukin 1\(\beta\) (n=8, \(P<0.01\)) and tumour necrosis factor \(\alpha\) (n=8, \(P<0.05\)), and significantly mitigated the change of the activities of antioxidant enzymes superoxide dismutase (n=8, \(P<0.001\)) and glutathione reductase (n=8, \(P<0.05\)). Emodin abolished the \textit{in vivo} prothrombotic effect of DEP in pial arterioles (n=6, \(P<0.01\)) and venules (n=6, \(P<0.001\)). Similarly, emodin prevented platelet aggregation \textit{in vitro} in whole blood (n=4-5, \(P<0.01\)), and the shortening of activated partial thromboplastin time (n=4, \(P<0.001\)) and prothrombin time (n=4, \(P<0.01\)) caused by DEP. \textbf{Conclusion:} We conclude that emodin treatment has consistently protected against DEP-induced impairment of vascular and cardiac homeostasis in mice. Our study provides experimental evidence that the use of functional food such as emodin, pending further studies, can be considered a useful agent and may have the potential to protect or mitigate the cardiovascular detrimental effects observed in people living in cities with high concentrations of particulate air pollution.
Introduction

Epidemiological studies reported that particulate air pollution not only has impact on the respiratory tract, but it has systemic and cardiovascular effects as well [1-3]. Acute exposure to particulate air pollution has been linked to a range of adverse cardiovascular events including hospital admissions with angina, myocardial infarction, and heart failure [4, 5]. Diesel exhaust emissions are a significant source of particulate air pollution in urban environments [4, 5]. As a consequence, exposure to diesel exhaust particles (DEP) have been used as models of particulate air pollution in experimental studies [4, 6].

The mechanisms underlying the acute cardiovascular effect of particulate air pollution are not fully elucidated [4, 7]. However, a key mechanism for their actions appears to be the generation of inflammation as a direct consequence of the ability of ambient particles to induce oxidative stress [4, 7, 8]. The last initiates proinflammatory actions related to atherogenesis [4, 7].

The antioxidants found in functional foods are of special interest in ameliorating oxidative stress in view of their safety and efficacy. Among these functional foods is emodin (1,3,8-trihydroxy-6-methylanthraquinone), which is an anthraquinone extracted from the root of the rhubarb plant (Rheum palmatum) that protects against oxidative stress in different models [9-12].

As cardiovascular toxicity of DEP is mediated, at least partially, by oxidative mechanisms [7], and emodin has protective or ameliorative effects against some experimental diseases and conditions involving inflammation and oxidative stress [9-12], we considered that it was of interest to evaluate the possible protective effect of emodin against DEP-induced cardiovascular complications in mice. To our knowledge, this is the first study on such an interaction.

Therefore, this study aims at investigating the possible protective effect of emodin on the acute (24h) cardiovascular effects of pulmonary exposure to DEP (0.5 mg/kg) in mice.

Material and Methods

**Diesel Exhaust Particles (DEP)**

The DEP were obtained from the National Institute of Standards and Technology (NIST, Gaithersburg, MD, USA), and were suspended in sterile saline (NaCl 0.9 %) containing Tween 80 (0.01 %). To minimize aggregation, particle suspensions were sonicated (Clifton Ultrasonic Bath, Clifton, New Jersey, USA) for 15 min and vortexed before their dilution and prior to intratracheal (i.t.) administration. Control animals received saline containing Tween 80 (0.01 %). These particles were previously analysed by transmission electron microscopy, and showed numerous small aggregates of carbonaceous particles less than 100 nm. Most of these aggregates were <1 μm in the largest diameter [13]. Geometric mean aerodynamic diameter of 215 nm generated from the same DEP material have been previously reported [14].

**Animals and treatments**

This project was reviewed and approved by the Institutional Review Board of the United Arab Emirates University, College of Medicine and Health Sciences, and experiments were performed in accordance with protocols approved by the Institutional Animal Care and Research Advisory Committee.

Male Tuck-Ordinary mice (UAEU, CMHS animal house) were housed in light (12-h light:12-h dark cycle) and temperature-controlled (22 ± 1°C) rooms. They had free access to commercial laboratory chow and were provided with tap water ad libitum.

The pulmonary exposure to DEP was achieved by intratracheal instillation (i.t.) [15]. Mice were first anesthetized with sodium pentobarbital ([60 mg/kg, intraperitoneal (i.p.)], placed supine with extended neck on an angled board. A Becton Dickinson 24 Gauge cannula was inserted via the mouth into the trachea. The DEP suspension (1 mg/kg)[15] or vehicle was instilled intratrachealy (i.t.) (50 μl) via a sterile syringe.
and followed by an air bolus of 100 µl. Emodin was given i.p. (4 mg/kg) twice 1 h before and 7 h following exposure to DEP or vehicle. This dosage of emodin used here is similar to dosage regimens used by others for other conditions [11]. The animals were randomly divided into four equal groups and were treated as follows:

- **Group 1 (n=8):** Normal saline given i.p. twice 1 h before and 7 h after the pulmonary exposure to vehicle;
- **Group 2 (n=8):** Normal saline given i.p. twice 1 h before and 7 h after the pulmonary exposure to DEP (1 mg/kg);
- **Group 3 (n=8):** Emodin (4 mg/kg) given i.p. twice 1 h before and 7 h after the pulmonary exposure to DEP (1 mg/kg);
- **Group 4 (n=8):** Emodin (4 mg/kg) given i.p. twice 1 h before and 7 h after the pulmonary exposure to DEP (1 mg/kg).

Twenty-four h following the pulmonary exposure to DEP or vehicle, various cardiovascular parameters were measured.

**Blood collection and cell count:** Mice were anesthetized intraperitoneally with sodium pentobarbital (60 mg/kg), and then blood was drawn from the inferior vena cava in EDTA (4 %). A sample was used for platelets, white blood cells and red blood cells counts using an ABX VET ABC Hematology Analyzer with a mouse card (ABX Diagnostics, Montpellier, France). The remaining blood was centrifuged for 15 min at 4°C at 900g, and the plasma samples obtained were stored at -80°C until further analysis.

**Measurement of markers of inflammation in the heart:** The same animals used for blood collection, were sacrificed by an overdose of sodium pentobarbital, and their hearts were quickly collected and rinsed with ice-cold PBS (pH 7.4). After that, half of the hearts were homogenized in 50 mM Tris buffer pH 7.4 containing 400 mM NaCl and 0.5 % Triton X-100 at 4°C. The homogenates were centrifuged for 10 min at 3000×g to remove cellular debris, and the supernatants were used for further analysis [16, 17]. The concentrations of tumour necrosis factor α (TNF α) and interleukin 1β (IL-1β) in the heart were determined using ELISA Kits (Duo Set, R & D systems, Minneapolis).

**Measurement of marker of oxidative stress in heart:** The second half of the hearts obtained from the above-mentioned animals were homogenized in 0.1M phosphate buffer pH 7.4 containing 0.15M KCl, 0.1mM EDTA, 1mM DTT and 0.1mM phenylmethylsulfonylfluoride at 4°C. The homogenates were centrifuged for 10 min at 3000×g to remove cellular debris, and supernatants were used for further analysis [18, 19]. Measurements of superoxide dismutase (SOD) and glutathione reductase (GR) in the heart tissue were performed according to previously reported techniques [12, 15, 17].

**Experimental cerebrovascular thrombosis model:** In a separate experiment (n=6 in each group), in vivo pial arteriolar and venular thrombogenesis was measured following DEP or vehicle administration with or without emodin treatment, according to a previously described technique [15, 19-21]. Briefly, the trachea was intubated after induction of anesthesia with urethane (1mg/g body weight, i.p.), and a 2F venous catheter (Portex, Hythe, UK) was inserted in the right jugular vein for the administration of fluorescein (Sigma, St. Louis, MO, USA). Thereafter, a craniotomy was first performed on the left side, using a microdrill, and the dura was stripped open. Only untraumatized preparations were used, and those showing trauma to either microvessels or underlying brain tissue were discarded. The animals were then placed on the stage of a fluorescence microscope (Olympus, Melville, NY, USA) attached to a camera and DVD recorder. A heating mat was placed under the mice and body temperature was raised to 37°C, as monitored by a rectal thermoprobe connected to a temperature reader (Physitemp Instruments, NJ, USA). The cranial preparation was moistened continuously with artificial cerebrospinal fluid of the following composition (mM): NaCl 124, KCl 5, NaH2PO4 3, CaCl 2.5, MgSO4.4, NaHCO3 23 and glucose 10, pH 7.3-7.4. A field containing arterioles and venules 15-20 µm in diameter was chosen. Such field was taped prior to and during the photochemical insult. The photochemical insult was carried out by injecting fluorescein (0.1ml/mouse of 5% solution) via the jugular vein, which was allowed to circulate for 30-40 sec. The cranial preparation was then exposed to stabilized mercury light. The combination produces endothelium injury of the arterioles and venules. This, in turn, causes platelet to adhere at the site of endothelial damage and then aggregate. Platelet aggregates and thrombus formation grow in size until complete arteriolar or venular occlusion. The time from the photochemical injury until full vascular occlusion (time to flow stop) in arterioles and venules were measured in seconds. At the end of the experiments, the animals were euthanized by an overdose of urethane.
In vitro assessment of platelet aggregation in whole blood: The platelet aggregation assay in whole blood was performed with slight modification as described before [17, 22]. After anesthesia, blood from separate animals (n=4-5) was withdrawn from the inferior vena cava and placed in citrate (3.8%), and 100-µl aliquots were added to the well of a Merlin coagulometer MC 1 VET (Merlin, Lemgo, Germany). The blood samples were incubated in vitro at 37°C with emodin (1µg/ml) in saline (0.9%) or saline alone for 3 min and then DEP (1µg/ml) or vehicle were added and incubated for 3 min. Each sample was then stirred for another 3 min. At the end of this period, 25-µl samples were removed and fixed on ice in 225 ml cellFix (Becton Dickinson, Franklin Lakes, NJ). After fixation, single platelets were counted in a VET ABX Micros with mouse card (ABX, Montpellier, France). Platelet aggregation was quantified by measuring the fall in single platelets counted due to aggregation induced by 1 µg DEP. The degree of platelet aggregation was expressed as a % of that obtained in untreated whole blood obtained from untreated mice.

In vitro assessment of activated partial thromboplastin time (aPTT) and prothrombin time (PT): The PT (n=4) was measured [15, 23, 24] on freshly collected, platelet-poor plasma with human relipidated recombinant thromboplastin (Recombiplastin; Instrumentation Laboratory, Orangeburg, NY, USA) in combination with a Merlin coagulometer [MC 1 VET (Merlin, Lemgo, Germany)]. Briefly, the plasma was incubated for 3 min at 37°C with emodin (1µg/ml) in saline (0.9%) or saline alone. After that, DEP (1µg/ml) or vehicle alone were added for another 3 min, and then PT was measured as previously described [15, 23, 24]. aPTT (n=4) was measured [15, 23, 24] with automated aPTT reagent [bioMerieux (Durham, NC, USA) using a Merlin coagulometer the MC 1 VET (Merlin, Lemgo, Germany)]. Briefly, the plasma was incubated for 3 min at 37°C, with emodin (1µg/ml) in saline (0.9%) or saline alone. After that, DEP (1µg/ml) or vehicle alone were added for another 3 min, and then aPTT was assessed as previously described [15, 23, 24]. Normal plasma used as reference for both the PT and aPTT was prepared by pooling equal portions of platelet-poor plasmas from the blood of four untreated mice.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism (version 5.03; GraphPad Software Inc, San Diego, CA, USA). The results are expressed as means ± SEM. Comparisons among groups were performed with one-way analysis of variance, followed by Newman-Keuls test. P values < 0.05 were considered to be significantly different.

Results

Number of circulating leukocytes and erythrocytes, haemoglobin concentration, and hematocrit

The number of circulating leukocytes was significantly (P<0.001) augmented by DEP compared with control group. The latter was significantly (P<0.001) abrogated by emodin treatment (Fig. 1A).

Likewise, compared with control group, the number of erythrocytes (P<0.01), haemoglobin concentration (P<0.05) and hematocrit (P<0.01) were significantly increased by DEP exposure. Emodin treatment significantly prevented the DEP effects and returned the number of erythrocytes (P<0.01), haemoglobin concentration (P<0.05) and hematocrit (P<0.01) to control values (Fig. 1B-D).

IL-1β and TNFα concentrations in heart tissue

Compared with control group, IL-1β (P<0.05) and TNFα (P<0.05) concentrations in heart homogenates were significantly increased after DEP exposure (Fig. 2A-B). Emodin treatment significantly prevented the increase of IL-1β (P<0.01) and TNFα (P<0.05) concentrations in heart.

SOD and GR activities in heart tissue

Figure 3 illustrates the effects of DEP on antioxidant activities SOD and GR, and the effects of emodin thereon. Compared with control group, DEP exposure caused a significant (P<0.01) decrease of the antioxidant SOD, indicating the occurrence of oxidative stress caused
Fig. 1. Leukocyte (A) and erythrocyte (B) numbers, (C) hematocrit and hemoglobin concentration (D), 24 h after intratracheal instillation of saline or diesel exhaust particles (DEP) with or without emodin treatment. Data are mean ± SEM (n=8).

Fig. 2. Interleukin 1β (IL1β, A) and tumour necrosis factor α (TNFα, B) concentrations in heart tissue, 24 h after intratracheal instillation of saline or diesel exhaust particles (DEP) with or without emodin treatment. Data are mean ± SEM (n=8).

Fig. 3. Superoxide dismutase (SOD, A) and glutathione reductase (GR, B) activities in heart tissue, 24 h after intratracheal instillation of saline or diesel exhaust particles (DEP) with or without emodin treatment. Data are mean ± SEM (n=8).
by DEP. The latter effect was completely reversed (P<0.001) by emodin treatment (Fig. 3A). DEP induced a significant (P<0.001) increase of the antioxidant GR, indicating that DEP could trigger adaptive responses that counterbalance the potentially damaging activity of oxygen radicals induced by DEP exposure. Interestingly, emodin treatment caused a significant (P<0.05) reversal of DEP-induced GR increase (Fig. 3B).

**Thrombosis in pial arterioles and venules**

Fig. 4 shows the effect of DEP on thrombotic occlusion time in pial arterioles and venules, and the effect of emodin treatment thereon. Pulmonary exposure to DEP induced a significant shortening of the thrombotic occlusion time in cerebral arterioles (P<0.01) and venules (P<0.01). Emodin treatment significantly abrogated DEP-induced prothrombotic effects in pial arterioles (P<0.01) and venules (P<0.001) (Fig. 4).

**Fig. 4.** Thrombotic occlusion time in pial arterioles (A) and venules (B), 24h after intratracheal instillation of saline or diesel exhaust particles (DEP) with or without emodin treatment. Data are mean ± SEM (n=6).

**Fig. 5.** *In vitro* platelet aggregation in whole blood after incubation with saline or diesel exhaust particles (DEP, 1µg/ml) with or without emodin (1µg/ml) treatment. Platelet aggregation was quantified by measuring the fall in single platelets counted due to aggregation induced by DEP. The degree of platelet aggregation was expressed as the percentage of that obtained in untreated whole blood obtained from untreated mice. Data are mean ± SEM (n=4-5).

**Fig. 6.** Activated partial thromboplastin time (aPTT; A) and prothrombin time (PT; B) *in vitro* after incubation with saline or diesel exhaust particles (DEP, 1µg/ml) with or without emodin (1µg/ml) treatment. Data are mean ± SEM (n=4).
Platelet aggregation in whole blood in vitro

Compared with control group, DEP caused a significant (P<0.01) platelet aggregation in vitro. Emodin treatment significantly (P<0.01) reversed the in vitro pro-aggregatory effect of DEP (Fig. 5).

aPTT and PT in vitro

Fig. 6 shows the impact of emodin treatment on the effects of DEP on aPTT and PT in vitro. Compared with control group, DEP induced a significant (P<0.001) shortening of aPTT. The latter effect was significantly (P<0.001) abrogated by emodin treatment (Fig. 6A). Similarly, DEP caused a small but significant (P<0.01) reduction in PT compared with control group. Emodin significantly (P<0.01) prevented the shortening of PT induced by DEP (Fig. 6B).

Discussion

In this work, we have shown that emodin treatment protected against DEP-induced increase in circulating leukocytes and erythrocytes, and cardiac inflammation and oxidative stress. We have also shown that emodin prevented coagulation events both in vivo and in vitro.

It is well established that the impact of particulate air pollution is not only limited to the lung but it can affect distant organs including heart, brain and kidney [7, 25, 26]. The latter effects are explained by 3 main hypotheses [7]. The first one relates to the effect of particles on their ability to impact the autonomic nervous system. Inhaled particles may affect the extrapulmonary sites through inflammatory mediators produced in the lungs and released into the circulation [7, 25, 26]. Moreover, several studies have shown that nanoparticles, owing to their small size, could avoid normal phagocytic defenses in the respiratory system and gain access to the systemic circulation and, therefore reach different extrapulmonary sites [27-30].

The dose of DEP used here 1 mg/kg (30µg/mouse) is analogous to the doses employed in former animal models of particulate air pollution exposure [31-33]. DEP was given to mice by i.t. instillation because it provides more accurate dosing, given that mice are nose breathers that filter most inhaled particles [34, 35].

Clinical and experimental studies have reported evidence for increase of leukocyte and erythrocyte numbers, and hematocrit following exposure to particulate air pollution [33, 36, 37]. In the present study, we showed that emodin significantly and markedly prevented the DEP-induced increase in leukocyte and erythrocyte numbers, hematocrit and hemoglobin concentrations. It has been recently demonstrated that emodin attenuates systemic inflammation in hyperlipidemic mice administrated with lipopolysaccharides [38].

While it is well established that emodin is a potent anti-inflammatory agent [38, 39], the protective effect of emodin on particulate air pollution induced cardiac inflammation has not been reported so far. In the present study, we show that emodin treatment abrogated the DEP-induced increase in cardiac proinflammatory cytokine TNFα and IL1β. It has been recently demonstrated that air pollution exposure induces myocardial inflammation characterized by increase of TNFα and IL1β and that dark chocolate provides cardioprotection [40].

The role of the oxidative stress in particulate-induced toxicity in different organs such as lung, liver and heart is well established [7]. Our data show that exposure to DEP caused a significant decrease of SOD in heart tissue suggesting a consumption of SOD during the breakdown of free radicals. Similar findings were observed in lung tissue of mice exposed to air pollution [16]. By contrast, GR activity was significantly increased suggesting that the increase in oxidative stress induced by DEP was accompanied by an increase of the activity of GR, indicating that the development of oxidative stress is accompanied by an adaptive response that counterbalances the potentially damaging activity of oxygen free radicals by antioxidant defense mechanisms [41, 42]. Interestingly, both changes in the activity of SOD
and GR were reversed by emodin treatment confirming its antioxidant capacity. The cardioprotective effect of emodin is greater at the level of antioxidants compared to that related to the release of proinflammatory cytokines. In line with our results, emodin has been reported to protect against cisplatin-induced nephrotoxicity and oxidative stress both in vivo and in vitro [9, 12].

We and others have previously reported that exposure to particulate air pollution cause prothrombotic complications [32, 33, 43, 44]. Our data show that DEP exposure caused a significant shortening of the thrombotic occlusion time in pial arterioles and venules, and that emodin treatment has consistently reversed the prothrombotic effects of DEP. This effect has, as far as we are aware, never been reported earlier. To verify whether and to what extent emodin displays similar effect in vitro, blood from untreated mice was treated with emodin, and then exposed to DEP. Our data show that emodin induced a significant reversal of the proaggregatory effect of DEP. This in vitro finding corroborates the in vivo protective effect of emodin on DEP-induced thrombotic complications in pial arterioles and venules.

Short-term exposure to air pollution is associated with changes in the global coagulation function, suggesting a tendency towards hypercoagulability [5]. Shortening of PT and aPTT have been reported by epidemiological and experimental studies [23, 45]. PT measures the formation of the fibrin clot through the activity of the extrinsic and common coagulation pathways, which involve the interaction of tissue factor and activated factor VII, in addition to factor X, factor V, prothrombin, and fibrinogen. aPTT measures the activity of the intrinsic and common pathways of coagulation. Our data show that DEP caused a significant shortening of the PT and aPTT, and that emodin treatment reversed these effects. These observations confirm the protective effect of emodin on DEP-induced hypercoagulability.

In conclusion, our results demonstrated that emodin significantly prevented DEP-induced cardiac inflammation and oxidative stress, and thrombotic complications in vivo and in vitro. These findings suggest that the use of functional food such as emodin, pending further studies, can be considered a useful agent and may have the potential to protect people of all ages against the cardiovascular events in cities with high concentrations of particulate air pollution.

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Disclosure Statement

None.

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