Macrophage Activation Is Responsible for Loss of Anticontractive Function in Inflamed Perivascular Fat

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Objective—The aim of this study was to determine whether macrophages dispersed throughout perivascular fat are crucial to the loss of anticontractive function when healthy adipose tissue becomes inflamed and to gain an understanding of the mechanisms involved.

Methods and Results—Pharmacological studies on in vitro small arterial segments from a mouse model of inducible macrophage ablation and on wild-type animals were carried out with and without perivascular fat using 2 physiological stimuli of inflammation: aldosterone and hypoxia. Both inflammatory insults caused a similar loss of anticontractive capacity of perivascular fat and increased macrophage activation. Aldosterone receptor antagonism and free radical scavengers were able to restore this capacity and reduce macrophage activation. However, in a mouse deficient of macrophages (CD11b-DTR), there was no increase in contractility of arteries following aldosterone incubation or hypoxia.

Conclusion—The presence and activation of macrophages in adipose tissue is the key modulator of the increase in contractility in arteries with perivascular fat following induction of inflammation. Despite multiple factors that may be involved in bringing about the vascular consequences of obesity, the ability of eplerenone to ameliorate the inflammatory effects of both aldosterone and hypoxia may be of potential therapeutic interest.

Key Words: hypoxia ■ microcirculation ■ obesity ■ inflammation

Obesity is set to reach epidemic levels in many countries,1 predisposing individuals to an increased risk of type II diabetes, hypertension, and the inevitable consequences of circulatory disease.2 The hallmark of this phenotype is an increase in the size of individual adipocytes as excess energy is stored, together with the recruitment and activation of macrophages and the release of cytokines as local inflammation ensues.3,4 The effect on the vasculature can be profound, with loss of vasodilator adiponectin bioavailability, increased tone,5,6 and the development of the metabolic syndrome.7

The cause of the inflammatory process in adipose tissue in obesity is uncertain. When present, it has been shown to lead to loss of bioavailability of dilator factors, including adiponectin.5–7 Two factors may be important: first, hypoxia has been shown to cause the release of inflammatory cytokines and loss of anticontractive function in adipose tissue, which can be restored using antagonists of tumor necrosis factor-α and interleukin-6.7 Second, increased production of aldosterone is reported in obesity-associated hypertension,8 which also can produce endothelial dys-

function9 and inflammation.10 Blocking the mineralocorticoid receptor leads to improvement of adipocyte function in murine obesity.11

The mechanisms responsible for the inflammatory process are unclear, but they could include activation of macrophages found dispersed throughout adipose tissue, the release of cytokines from adipocytes, or both cell types being involved. Therefore, the aim of this study was to determine which of these is crucial to the loss of anticontractive function when healthy perivascular fat becomes inflamed, thereby influencing tone, and to gain an understanding of the systems involved. Using a series of pharmacological studies on in vitro arterial segments and vessels from a mouse model of inducible macrophage ablation,12 we show that aldosterone and hypoxia can mimic the effect of reproducing an inflammatory phenotype in adipose tissue and a loss of anticontractive function. We show also that it is macrophage activation that is responsible if this does not occur, no loss of anticontractive activity is seen. We demonstrate that adipose tissue integrity can be maintained by the aldosterone.
antagonist eplerenone, which may suggest a new role for such agents in improving the vascular effects of obesity.

Methods

An expanded Supplemental Methods section, available online at http://atvb.ahajournals.org, gives detailed information on the mouse model, materials, pharmacology, and histology protocols.

Animals

Procedures were performed in accord with Institutional Guidelines and the United Kingdom Animals (Scientific Procedures) Act of 1986. Healthy male Wistar rats (aged 12 to 15 weeks), macrophage-deficient (CD11b-DTR) mice bred onto a FVB/N background, and FVB/N wild-type (WT) controls were killed by stunning followed by cervical dislocation. The mesenteric bed was immediately removed and placed in ice-cold physiological salt solution (PSS); first-order arteries were identified (diameter, 250 to 300 μm) and partly dissected clean of fat, whereas the other part was left with surrounding fat intact.

Inducing Macrophage Depletion

Macrophage ablation of CD11b-DTR mice (aged 16 to 20 weeks) was induced by intraperitoneal injection of diphtheria toxin at 20 ng/g. Mice were injected on days 0, 2, and 4 and euthanized on day 5.

Pharmacological Assessment of Contractility

Mesenteric small arteries (internal diameter, <275 μm/L) with and without intact perivascular fat were studied using a wire myograph at 37°C and bubbled with 95% air/5% CO2 to saturate the vessel bath while maintaining pH 7.4, as described previously. Arterial viability was assessed by constriction with high-potassium PSS, and endothelial integrity was determined by addition of 1×10⁻⁵ M acetylcholine. Constriction of arteries with 60 mmol/L high-potassium PSS was performed after each dose response curve to norepinephrine (1×10⁻⁶ to 1×10⁻³ mol/L).

Contractility of arteries was investigated following 10-minute and 3-hour incubations with aldosterone (5 nmol/L), with and without eplerenone (5 μmol/L), and following the induction of experimental hypoxia (2.5 hours, 95% N₂/5% CO₂) with and without eplerenone (5 μmol/L). Arteries incubated with aldosterone for 10 minutes or 3 hours were also incubated with superoxide dismutase (80 U/mL) and catalase (120 U/mL) to ascertain the mechanism by which aldosterone may cause its effects. The involvement of the endothelium in vascular reactivity was assessed following mechanical removal of the endothelium by passing a wire through the lumen, and denudation was confirmed by addition of acetylcholine (1×10⁻⁵ mol/L) to vessels preconstricted with 60 mmol/L high-potassium PSS.

Histological Characterization of Perivascular Fat

To characterize the effect of aldosterone and its antagonists on macrophage activation, segments of perivascular fat from the mesenteric bed of Sprague-Dawley rats were incubated as part of wire myography experiments at 37°C and bubbled with 95% air/5% CO₂. Samples were fixed using 4% paraformaldehyde and embedded in paraffin wax. Specimens were cut into 5 μmol/L sections and stained using the Perls’ Prussian blue technique for the detection of iron-positive cells, such as activated macrophages. Five sections from each segment were analyzed semiquantitatively (Lucia Image Analysis software, Nikon), and levels of iron storage were graded by counting the number of positive cells per field of view.

Statistical Analysis

Data are presented as mean±SEM. Differences in response to norepinephrine were expressed as a percentage of constriction to high-potassium PSS and analyzed using a 2-way ANOVA followed by Bonferroni post hoc test at each dose response point. Differences in staining for iron-loaded positive macrophages were tested using a Wilcoxon signed rank test, comparing column medians to that observed in untreated, immediately fixed perivascular fat. Probability values <0.05 were considered significant. Analyses were performed with GraphPad Prism, version 3.00, for Windows (GraphPad Software).

Results

Pharmacological Assessment of Vessel Contractility in Rat Mesenteric Arteries

The presence of perivascular fat was associated with a significant anticontractile effect (P<0.0001, n=36), which was lost following incubation with aldosterone for both 10 minutes (P<0.0001, n=25) (Figure 1A) and 3 hours (P<0.0001, n=7) (Figure 1B).

Aldosterone and Hypoxia Increase Oxidative Stress

Induction of experimental hypoxia for 2.5 hours (95% N₂/5% CO₂) caused a loss of the anticontractile effect (P<0.0001, n=15) similar to that observed following aldosterone incubation (Figure 2A). The presence of the free radical scavengers during 3-hour aldosterone incubation was able to prevent the loss of the anticontractile capacity of perivascular fat (P<0.0001, n=5) suggesting a common pathway for aldosterone and hypoxia (Figure 2B).
the free radical scavengers during the 10-minute aldosterone incubation was associated with a partial reduction in contractility that was not significant (Supplemental Data). Experimental hypoxia and aldosterone incubation in combination was associated with a similar contractile response compared with the individual inflammatory stimuli (data not shown).

The Effects of Aldosterone and Hypoxia Can Be Prevented Using a Mineralocorticoid Receptor Antagonist

The aldosterone receptor antagonist eplerenone was able to reverse the effect of 10-minute and 3-hour aldosterone incubations to levels comparable to those of arteries with healthy fat (fat + 10-minute aldosterone versus fat + 10-minute aldosterone + eplerenone: \(P < 0.0001\), \(n = 7\); fat + 3-hour aldosterone versus fat + 3-hour aldosterone + eplerenone: \(P < 0.0001\), \(n = 5\)) (Figure 3A and 3B). In addition, incubation with eplerenone during hypoxia (\(n = 7\)) was able to restore the anticontractile capacity of the surrounding fat in the absence of any supplemented aldosterone (\(P = 0.044\)) (Figure 3C).

No statistical differences between ED\(_{50}\) for each treatment were observed, suggesting that any variation in response was not as a result of changes in sensitivity of the artery to norepinephrine. Although treatment of arteries without fat had some effect on contractility, none of these were significant. In addition, incubation of arteries with antagonist alone under normoxic conditions had no significant effect on contractility (Supplemental Materials).

Histological Characterization of Rat Mesenteric Perivascular Fat

Differences in the grading of iron-loaded cells were observed between sample groups. The number of positive cells was increased following 3-hour aldosterone-treated fat samples compared with levels in tissue in PSS alone for the same time period (3-hour PSS: 7.3 \(\pm\) 0.6, \(n = 5\), versus 3 hours of aldosterone: 19.4 \(\pm\) 0.3, \(n = 5\), \(P = 0.04\)), and there was a moderate but not significant increase in macrophage activation following 10-minute aldosterone treatment (10-minute PSS: 6.6 \(\pm\) 0.4, \(n = 5\), versus 10-minute aldosterone: 14.7 \(\pm\) 0.4, \(n = 5\), \(P = \text{not significant}\)). Eplerenone was able to prevent the increase in the number of iron-loaded macrophages observed following 3-hour aldosterone incubation (11.0 \(\pm\) 0.3, \(n = 5\)) to levels similar to those incubated for the same time period in PSS (6.9 \(\pm\) 0.4, \(n = 5\)). Similarly, hypoxia was associated with an increase in the number of iron-loaded macrophages observed following 3-hour aldosterone incubation (11.0 \(\pm\) 0.3, \(n = 5\)) to levels similar to those incubated for the same time period in PSS (6.9 \(\pm\) 0.4, \(n = 5\)). Similarly, hypoxia was associated with an increase in the number of iron-loaded macrophages (23.0 \(\pm\) 0.5, \(n = 5\), \(P = 0.0156\)), which was not seen when eplerenone (11.0 \(\pm\) 0.8, \(n = 4\), \(P = 0.3750\)) was included (Figure 4).

**Figure 2.** Aldosterone (Ald) and hypoxia increase oxidative stress. A, 2.5 hours of experimental hypoxia (\(n = 15\)) caused an increase in contractility of arteries surrounded by fat compared with those in normoxia (\(n = 36\), *\(P < 0.0001\)). B, The free radical scavengers superoxide dismutase (SOD) (80 U/mL) and catalase (CAT) (120 U/mL) were able to reverse the effects of 3-hour aldosterone incubation (\(n = 5\), †\(P < 0.05\)). KPSS indicates high-potassium PSS; NE, norepinephrine.

**Figure 3.** The effects of aldosterone (ald) and hypoxia can be prevented using a mineralocorticoid receptor antagonist. A, Eplerenone (5 \(\mu\)mol/L, \(n = 7\)) was able to fully restore the anticontractile capacity of the perivascular fat following 10-minute aldosterone incubation (\(n = 25\), *\(P < 0.0001\)). B, Eplerenone (EP; 5 \(\mu\)mol/L) (\(n = 5\)) also reduced contractility compared with arteries surrounded by perivascular fat incubated with aldosterone for 3 hours (\(n = 7\), *\(P < 0.0001\)). C, The loss of anticontractile capacity of perivascular fat associated with experimental hypoxia was absent following incubation with eplerenone (5 \(\mu\)mol/L, \(n = 5\), #\(P < 0.01\)). KPSS indicates high-potassium PSS.
Contractility in the Mesenteric Arteries of a Macrophage-Deficient Mouse Model

Pharmacological Assessment of Vessel Contractility in the Mesenteric Arteries of a Macrophage-Deficient Mouse Model

The response of arteries from WT and macrophage-deficient (CD11b-DTR) mice to norepinephrine were not significantly different both in the absence and presence of perivascular fat (no fat: WT versus CD11b-DTR, n=3, P=not significant; fat: WT versus CD11b-DTR, n=3, P=not significant) (Figure 5A and 5B). Both 10-minute and 3-hour aldosterone incubation caused a loss of the anticontractile capacity of adipose tissue in arteries from WT animals (fat versus 10-minute aldosterone: n=3, P=0.0368; fat versus 3-hour aldosterone: n=3, P=0.049) (Figure 5C and 5E) although this was not significant in arteries from CD11b-DTR mice (Figure 5D and 5F). Eplerenone rescued the loss of anticontractile capacity of perivascular fat in WT arteries (10-minute aldosterone versus 10-minute aldosterone + eplerenone: n=3, 0.0227; 3-hour aldosterone versus 3-hour aldosterone + eplerenone, n=3, P<0.0001) (Figure 5C and 5E). Incubation of arteries from CD11b-DTR animals with aldosterone and eplerenone was not associated with a significant change in final response to norepinephrine; however, there were some differences at lower norepinephrine doses (P=0.0003) compared with the response of arteries from CD11b-DTR animals surrounded by perivascular fat at the same points (Figure 5D and 5F). Following the induction of experimental hypoxia, the loss on the anticontractile effect of perivascular adipose tissue was less in arteries from CD11b-DTR animals compared with WT controls (P=0.0001). The effects of hypoxia were reversed in WT following incubation with eplerenone (P<0.001), which was not observed in arteries from CD11b-DTR animals (Figure 5G and 5H).

No significant differences were observed in ED₅₀ between each treatment group in arteries taken from WT and CD11b-DTR animals.

Discussion

The important finding of this study is that the activation of macrophages found dispersed throughout adipose tissue is responsible for the loss of anticontractile activity when healthy perivascular fat is subjected to proinflammatory insults. We demonstrated that macrophage activation is observed when either aldosterone or the induction of hypoxia is used as a stimulus and that both of these can be ameliorated using the mineralocorticoid receptor antagonist eplerenone.

Obesity is associated with low levels of adipose tissue inflammation,3,4 which has been shown to have detrimental effects on the function of perivascular fat.7 Data from such tissue stained using Perls’ Prussian blue for iron-loaded adipocytes16 identified activation, which occurs within 10 minutes of the application of aldosterone and is also seen when the tissue is rendered hypoxic. Aldosterone application has previously been shown to cause an increase in the expression of the classical activation marker Tnfα in cultured peritoneal macrophages; this increase in expression is prevented by eplerenone.17 To determine the functional significance of the observed macrophage activation and to understand the relative contribution to the loss of anticontractile function of perivascular fat, we used a mouse model of inducible macrophage deficiency.13,18,19 It has been shown that following intraperitoneal injection of diphtheria toxin in these mice, there is a marked ablation of macrophages in the surrounding tissues, including the fatty tissue of the mammary gland.13,20 Our data demonstrate that the loss of anticontractile activity was severely attenuated in vessels from CD11b-DTR mice compared with WT animals, indicating that the presence and activation of macrophages is crucial to the increases in contractility seen when inflammation is induced. It is of interest that the recruitment of macrophages following mineralocorticoid/salt administration has previously been suggested as the key mechanism behind the development of cardiac fibrosis mediated by an increase in oxidative stress.21,22 In this context, it is also worth noting that the effects of our inflammatory stimuli could be prevented by the use of catalase and superoxide dismutase acting as free radical scavengers, implying that the common pathway to
loss of anticontractile function is an increase in oxidative stress. This reinforces our previous data, where Greenstein et al showed that the detrimental effects of experimental hypoxia could be rescued using free radical scavengers. Of the 2 methods used to induce inflammation, aldosterone was applied to the organ bath for shorter and longer time periods to examine the effects of nongenomic and genomic influences on vascular function. Our studies clearly demonstrate that aldosterone was able to bring about its effects by both mechanisms, and we confirmed that this was due to increased oxidative stress via cross-talk between the classical genomic and nongenomic pathways, although we accept that no inhibitors of transcription or other markers of its effects were used. The ability to rescue the anticontractile function using free radical scavengers again points to both hypoxia and aldosterone using a final common pathway of increasing oxidative stress. However, it is of possible clinical relevance that both the effects of aldosterone and hypoxia could be prevented with the application of the mineralocorticoid receptor antagonist eplerenone. In this respect, our data support a previous study that has shown that aldosterone contributes to oxidative stress in vascular smooth muscle cells by genomic and nongenomic mechanisms. Furthermore, as no supplementary aldosterone was added to the organ bath during the induction of hypoxia, we can conclude that this inflammatory stimulus induces changes that are associated with activation of the mineralocorticoid receptor. Cultured adipocytes are not known to express cytochrome aldosterone synthase mRNA, critical in the synthesis in the new aldosterone, thereby reinforcing a cross-talk relationship between perivascular fat and smooth muscle cells. This is the first time this has been observed in adipose tissue, although other studies have shown that eplerenone can have protective effects in conditions associated with elevated reactive oxygen species, including atherosclerosis, neointima formation following coronary stenting, and abnormal left ventricular relaxation in mild hypertension, independent of aldosterone.

Figure 5. Comparison of contractility of arteries to norepinephrine (NE) with perivascular fat from macrophage-deficient (CD11b-DTR) and WT mice. Arteries were incubated with aldosterone (Ald; 5 nmol/L) for 10 minutes or 3 hours with or without eplerenone (EP; 5 μmol/L) and were exposed to experimental hypoxia (95% N2/5%CO2) for 2.5 hours with or without eplerenone (5 μmol/L, n = 3). A, Healthy fat caused a reduction in contractility to norepinephrine compared with WT arteries without fat. B, A similar effect was observed in arteries from CD11b-DTR mice (*P < 0.001). C, 10-minute incubation with aldosterone caused a loss of the anticontractile effect of perivascular fat in WT arteries (*P < 0.001), which was reversed by eplerenone. D, Aldosterone with or without eplerenone had no significant effect on the anticontractile effect of perivascular fat on arteries from CD11b-DTR mice. E, 3-hour incubation with aldosterone caused a loss of the anticontractile effect of perivascular fat in WT arteries, which was reversed by eplerenone. F, 3-hour aldosterone incubation with or without eplerenone had no significant effect on the anticontractile effect of perivascular fat on arteries from CD11b-DTR mice. G, Hypoxia caused an increase in contractility of WT arteries surrounded by fat compared with those in normoxia, which was reversed by eplerenone. H, Hypoxia with or without eplerenone had no significant effect on arteries surrounded by fat from CD11b-DTR mice. KPSS indicates high-potassium PSS.
A number of mechanisms by which hypoxia and macrophage activation may cause an increase in mineralocorticoid-associated vascular contractility have been proposed, including changes in 11βHSD-2. Both type 1 and type 2 dehydrogenase enzymes have been identified in adipose tissue, and the equilibrium of their activity ensures that the higher plasma concentrations of glucocorticoids are converted to 11-keto derivatives and therefore have a lower affinity for the mineralocorticoid receptor. Hypoxia downregulates the expression of the type 2 enzyme, generating more of the active cortisol/corticosterone that is available to activate the receptor; this provides a potential explanation of why eplerenone is able to reverse the effects of hypoxia.38–40 Despite multiple factors that may be involved in bringing about the vascular consequences of obesity, the ability of eplerenone to ameliorate the inflammatory effects of both aldosterone and hypoxia may be of potential therapeutic interest. Trials of this agent in these clinical phenotypes may be of particular interest in terms of introducing a new way of addressing the prevention of obesity-related morbidity and mortality beyond the recognized reductions in blood pressure observed with such agents.

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Disclosures
None.

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SUPPLEMENT MATERIAL

Methods

Materials
Aldosterone, acetylcholine, norepinephrine, superoxide dismutase, catalase and histology chemicals were purchased from Sigma-Aldrich (Gillingham, UK). Eplerenone was provided by Pfizer Inc. Wyeth, UK. Salts for physiological salt solutions were obtained from VWR (Lutterworth, UK).

Macrophage deficient mouse model
Macrophage deficient mice were a kind gift from Prof Else, Faculty of Life Sciences, University of Manchester. The CD11b-human DT receptor (DTR) transgene was used to generate mice on the FVB/N background using conventional techniques. RT-PCR was used to confirm transgene expression; primer sequences used were AAGATCCGCCACAACATCG for the 5’ primer and GCAGCTCTAGGGTTGGATTTCG for the 3’ primer. No PCR product could be amplified from genomic DNA. Injection of mice using diphtheria toxin (DT) conditionally ablates monocytes/macrophages in these mice through binding to the hbEGF receptor (heparin-binding epidermal growth factor-like growth factor) which following internalisation inhibits protein synthesis.1

Pharmacological assessment of vessel contractility
Effects of short incubation with aldosterone
As the earliest genomic effects of aldosterone have not been observed before 30 minutes following application2,3, the more rapid non-genomic effect on resistance arteries was investigated by a short incubation with aldosterone. Arteries with and without perivascular fat were incubated with increasing concentrations of aldosterone (1x10^-9 - 1x10^-8 mol/L) for 10 minutes, after each aldosterone concentration a dose response curve to norepinephrine was constructed. The concentration at which the maximum effect on contractility was observed was used for further investigations.

The effect of the aldosterone antagonist eplerenone on contractility was assessed. Arteries were initially incubated with the antagonist alone to determine whether there
was any side effect in the absence of aldosterone. Arteries were then incubated with aldosterone and eplerenone (5µmol/L) for 10 minutes, and a dose response curve to norepinephrine was constructed.

Effects of long incubation with aldosterone
In order to investigate the effect of longer incubation with aldosterone, thus allowing some of the genomic changes associated with aldosterone to occur, arteries with and without perivascular fat were incubated with aldosterone (5nmol/L) for 3 hours. Addition of eplerenone (5µmol/L) was also assessed over the 3 hour period.

The effect of free radical scavenging during aldosterone incubation
To ascertain the mechanism by which aldosterone may cause its effects, arteries were also incubated with superoxide dismutase (80U/mL) and catalase (120U/mL) during both the 10 minute and 3 hour aldosterone incubation period (5nmol/L). The free radical scavenger enzymes protect against the effects of free radicals which may be generated by the action of aldosterone establishing the contribution of this pathway to the effects of aldosterone in the vasculature.

The effect of hypoxia on the anticontractile capacity of perivascular fat
As some of the effects of aldosterone are thought to be mediated via the production of reactive oxygen species (ROS), the response to experimental hypoxia was assessed. Arteries with and without perivascular fat were incubated for 2.5 hours in physiological salt solution (PSS) at 37°C bubbled with 95%N₂/5%CO₂. Hypoxia was determined using an oxygen probe, levels of oxygen tension of 35 mmHg were achieved within 10 min and were stable throughout hypoxia, after which contractility was determined. Application of eplerenone (5µmol/L) during the time of hypoxia was investigated to establish whether the antagonist was able to protect the tissue from the effects of experimental hypoxia.

Effects of endothelium on the anticontractile capacity of perivascular fat
To determine the involvement of the endothelium in arterial response, arteries were denuded mechanically by passing a wire through the lumen of the vessel. Contractile responses to high potassium solution were assessed before and after endothelium removal to determine vessel integrity, followed by addition of acetylcholine (1x10⁻⁶ mol/L).
5 mol/L) to confirm vessel denudation. The effect of 10 minute and 3 hour aldosterone incubation (± eplerenone or SOD/CAT) and experimental hypoxia (± eplerenone or SOD/CAT) on arteries with and without perivascular fat were then investigated.

**Histological characterization of perivascular fat tissue**

*Characterizing macrophage activation*

To characterize the effect of aldosterone, hypoxia and the aldosterone antagonist eplerenone on macrophage activation, segments of perivascular fat from the mesenteric bed of male wistar rats were incubated at 37ºC bubbled with 95% air /5%CO₂ as for wire myography experiments. The effect of 10 minute or 3 hour aldosterone incubations and 2.5 hours experimental hypoxia, both with and without eplerenone was compared with activated macrophage levels in tissue incubated with PSS for the same time period. Samples were fixed using 4% paraformaldehyde (pH 7.4) at 4ºC for 24 hours, dehydrated though an ethanol series, treated with xylene, embedded in paraffin wax and then cut into 5µM sections. Deparaffinised sections were stained using the Perl’s Prussian blue technique for the detection of iron positive cells such as activated macrophages. Briefly sections were incubated in 2% aqueous potassium ferricyanide-hydrochloric acid solution for 15 minutes and counterstained using saffrinin-O. Free ferric ions are released by treatment with the hydrochloric acid, which react with potassium ferrocyanide to form insoluble ferric ferrocyanide, which is blue in colour. Five sections from each segment were analysed semi-quantitatively using Lucia Image Analysis software, Nikon, UK). Levels of iron storage were graded 0-10 based on the number of positive cells per field of view and referred to as the macrophage abundance value. Fields of view were selected at random to ensure unbiased quantification. Cumulative analysis showed five images were sufficient to be representative of the mean value within the sample.
Results

*Aldosterone and hypoxia have no significant effect on arteries in the absence of perivascular fat*

Induction of experimental hypoxia for 2.5 hours (95% N\textsubscript{2}/ 5% CO\textsubscript{2}) had no effect on the contractility of arteries when perivascular fat was removed (n=15, P=0.29) (Figure I), similar to that observed following aldosterone incubation (10 minute aldosterone n=25, P=0.95, 3 hour aldosterone: n=x, P=0.07) (Figure II). The presence of free radical scavengers or eplerenone during 3 hour aldosterone incubation (Figure III) and experimental hypoxia (Figure I) also had no significant effect on contractility in these arteries (10 minute aldosterone + eplerenone: P=0.52, 3 hour aldosterone: P=0.29, 3 hour aldosterone + SOD/CAT: P=0.54, hypoxia: 0.56).

*Free radical scavengers are unable to reverse the effects of short term aldosterone incubation*

Incubation of arteries with the free radical scavengers during 10 minute aldosterone was associated with a partial reduction in contractility which was not significant (Figure IV).

*The anticontractile capacity of perivascular fat is independent of the endothelium*

Denudation of arteries, both with and without perivascular fat had no effect on the contractile response compared to the same arteries pre-denudation (Figure V). The anticontractile effect of perivascular fat was lost upon both 10 minute and 3 hour incubation with aldosterone and 2.5 hours of experimental hypoxia (10 minute aldosterone: P=0.036, n=4; 3 hour aldosterone: P<0.0001, n=4; hypoxia: P=0.033, n=4) (Figure VI). Eplerenone was only able to part restore the anticontractile capacity of the perivascular fat (10 minute aldosterone vs. 10 minute aldosterone + eplerenone: P=0.42, n=4; 3 hour aldosterone vs. 3 hour aldosterone + eplerenone: P=0.06, n=4; hypoxia: P=0.18, n=4) although this was not significant (Figure VII). Incubation with the free radical scavengers superoxide dismutase and catalase during inflammatory stimuli was caused a significant reduction in the loss of effect associated with 3 hour aldosterone alone (P=0.0003, n=4), although there was a part rescue effect in hypoxia, this was not significant (P=0.1, n=4) (Figure VIII).
Supplementary figures

Figure I. The effect of hypoxia on the contractility of healthy rat mesenteric arteries in the absence of perivascular fat. Experimental hypoxia for 2.5 hours (n=15) had no significant effect on contractility of arteries compared with arteries without perivascular fat (n=36) (P=0.29). Incubation with eplerenone (n=5) throughout hypoxia also had no significant effect on contractility (P=0.56)
Figure II. The effect of aldosterone incubation on the contractility of healthy rat mesenteric arteries in the absence of perivascular fat. A. Aldosterone incubation for 10 minutes (n=25) had no significant effect on contractility of arteries compared with arteries without perivascular fat (n=36) (P=0.95). Incubation with eplerenone (n=7) throughout aldosterone incubation also had no significant effect on contractility (P=0.52). B. Aldosterone incubation for 3 hours (n=7) had no significant effect on contractility of arteries compared with arteries without perivascular fat (n=36) (P=0.07). Incubation with eplerenone (n=7) throughout aldosterone incubation also had no significant effect on contractility (P=0.29)
Figure III. The effect of free radical scavenging on the contractility of healthy rat mesenteric arteries in the absence of perivascular fat. A. Aldosterone incubation for 10 minutes (n=25) had no significant effect on contractility of arteries compared with arteries without perivascular fat (n=36) (P=0.95). Incubation with SOD/CAT (n=5) throughout aldosterone incubation also had no significant effect on contractility (P=0.32). B. Aldosterone incubation for 3 hours (n=7) had no significant effect on contractility of arteries compared with arteries without perivascular fat (n=36) (P=0.07). Incubation with superoxide dismutase (SOD) (80U/mL) and catalase (CAT) (120U/mL) (n=5) throughout aldosterone incubation also had no significant effect on contractility (P=0.54).
Figure IV. The effect of free radical scavenging on the contractility of healthy rat mesenteric arteries in the presence of perivascular fat. Incubation with SOD/CAT throughout the 10 minute aldosterone incubation had no significant effect on contractility (P=0.87, n=4).
Figure V. Denudation of arteries has no effect on the contractile response of arteries to norepinephrine in the presence or absence of perivascular fat. No significant difference was observed in contractility in arteries with or without perivascular fat following denudation (No fat + endothelium vs. no fat denuded: P=0.83; fat + endothelium vs. fat denuded: P=0.24, n=4).
Figure VI. The effect of aldosterone and hypoxia on the anticontractile capacity of perivascular fat in denuded rat mesenteric arteries. A. Perivascular fat caused a reduction in contractility to norepinephrine compared with denuded arteries without fat (P=0.0002, n=4). Incubation with aldosterone (5nmol/L) for 10 minutes (n=4) caused a loss of this effect (*=P=0.036). B. A similar increase in contractility was observed in arteries incubated with aldosterone (5nmol/L) for 3 hours (n=4) (†=P<0.0001). C. Experimental hypoxia for 2.5 hours (95%N₂/5%CO₂) was associated with an increase in contractility in arteries with perivascular fat (P=0.033, n=4) *=P<0.05, †=P<0.001.
Figure VII. The effect of eplerenone on denuded arteries following incubation with inflammatory stimuli in denuded arteries. Eplerenone is unable to restore the anticontractile effect of perivascular fat lost following 10 minute incubation with aldosterone (P=0.42, n=4). B. Eplerenone part restored the anticontractile effect of perivascular fat lost following 3 hour aldosterone incubation although this was not significant (P=0.06, n=4). C. The loss of perivascular function associated with hypoxia was not restored by eplerenone (P=0.18, n=4).
Figure VIII. The effect of free radical scavenging in denuded arteries following incubation with inflammatory stimuli in denuded arteries. SOD/CAT is unable to restore the anticontractile effect of perivascular fat lost following 10 minute incubation with aldosterone (P=0.68, n=4). B. SOD/CAT restored the anticontractile effect of perivascular fat lost following 3 hour aldosterone incubation (P=0.003, n=4). C. The loss of perivascular function associated with hypoxia was part restored by eplerenone although this was not significant (P=0.1, n=4).
References


