cGMP-dependent protein kinase (PKG) mediates the anticontractile capacity of perivascular adipose tissue

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1. Introduction

There is accumulating evidence that adipocytes that surround blood vessels play an important modulating role in arterial tone: healthy perivascular adipose tissue (PVAT) is highly metabolically functional and acting in a paracrine manner produces a number of dilators including adiponectin. Healthy PVAT exerts an anticontractile effect on vessels via a number of phosphorylation events including vasodilator-stimulated phosphoprotein (VASP) and acting via BKCa channels in vascular smooth muscle has been described, but the exact intracellular downstream mechanisms involved are unknown. Previously, we have recreated the inflammatory environment in an ex vivo system by inducing experimental hypoxia.⁴

cGMP-dependent protein kinase (PKG) is considered to play a significant role in the relaxation of smooth muscle.⁵ Two different PKG isoforms are expressed in mammals, but only cGMP dependent protein kinase I (PKGI) is highly expressed in smooth muscle and can be found in other cells of the vasculature including adipocytes.⁶ Induction of PKGI occurs downstream of nitric oxide activation of soluble guanylyl cyclase (sGC) and increases in cGMP, and the resulting relaxation is likely due to a number of phosphorylation events including vasodilator-stimulated phosphoprotein, AMPK, and BKCa channels.⁷ The absence of PKG is associated with hypertension.⁸ Furthermore, PKG has been implicated in obesity because recent work has shown that

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the intake of a high-fat diet is associated with a reduction in adipose tissue PKG expression and so provides a link between obesity and the subsequent loss of the anticontractile capacity of PVAT and development of hypertension.

Therefore, we investigated the role of PKG in mediating healthy PVAT function and in response to experimental hypoxia using a series of pharmacological studies on in vitro arterial segments and vessels from a PKG knockout mouse model. We report here that PKG is crucial for the release of relaxing factor(s), including adiponectin, from PVAT and also in the actions of such factors on the vasculature.

2. Methods

Detailed materials and methods are available in Supplementary material online.

2.1 Animals

Procedures were performed in accordance with the United Kingdom Animals (Scientific Procedures) Act of 1986 and Institutional Guidelines and conform to the Directive 2010/63/EY of the European Parliament. Male wild-type (PKG+/−) and PKG-I-deficient (PKG−/−) mice, which were kindly provided by Prof. Hofmann (Munich, Germany) and Prof. Lukowski (Tuebingen, Germany) to Dr ME Werner. B6.129-Adipoqtm1Chan/J adiponectin-deficient mice (adipo−/−) were purchased from The Jackson Laboratory (Maine, USA) and used at 10 weeks. C57Bl/6j mice were purchased from Harlan, UK and used as controls for the adipo−/− mice. Mice were killed by cervical dislocation.

2.2 Pharmacological assessment of contractility

First-order mesenteric arteries with and without intact PVAT were studied in PKG+/− and PKG−/− mice under normal oxygen tension and experimentally induced hypoxia (2.5 h dislocation. The contractility of arteries was investigated in PKG+/− and PKG−/− mice under normal oxygen tension and hypoxia (2.5 h dislocation.

2.3 Wire myography

The contractility of arteries was investigated in PKG−/− and PKG+/− mice under normal oxygen tension and experimentally induced hypoxia (2.5 h). Arteries were pre-constricted with 1 × 10−5 mmol/L norepinephrine and allowed to reach a stable level of constriction. The total bath solution (6 mL) from the recipient’s artery was then replaced with that of the donor’s artery (6 mL) and change in tension recorded.

The role of PKG in the transferrable anticontractile capacity of PVAT was determined using pharmacological tools, ODQ and DT-2, and small artery segments from PKG−/− and PKG+/− animals. Different combinations were used to establish at which point PKG is involved in PVAT-associated relaxation.

2.4 Organ bath solution transfer experiments

Solution transfer experiments were employed to study the role of PKG in the release of relaxing factors from PVAT. Arteries with PVAT intact were used as donors, and those devoid of PVAT were used as recipients. Arteries were pre-constricted with 1 × 10−5 mmol/L norepinephrine and allowed to reach a stable level of constriction. The total bath solution (6 mL) from the recipient’s artery was then replaced with that of the donor’s artery (6 mL) and change in tension recorded.

2.5 Histological characterization of PVAT

Histological detection of adiponectin and superoxide dismutase was performed in PVAT samples from PKG+/− and PKG−/− mice under conditions of normal oxygen tension and hypoxia ± ANP. Adipocyte size was derived from haematoxylin and eosin staining and measured using the Image J analysis software.

2.6 Statistical analysis

Data are presented as means ± SEM. Differences in response to norepinephrine were expressed as a percentage of constriction to high potassium (60 mM) PSS (KPSS) and analysed by the two-way ANOVA and Bonferroni post hoc test. P-values of <0.05 were deemed significant. GraphPad Prism, version 3.00 for Windows, was used for analysis of data.

3. Results

3.1 Healthy PVAT has an anticontractile effect which is endothelium-dependent

In line with previously published results, healthy PVAT had an anticontractile effect on arteries from PKG+/− (wild-type) mice (P < 0.001, n = 13) (Figure 1A). The anticontractile effect was due to the secretion of a soluble factor or factors, as transfer of organ bath solution between pre-constricted (1 × 10−5 M norepinephrine) vessels + PVAT to vessels− PVAT resulted in a significant relaxation (Δ tension: 0.25 ± 0.034 mN/mm, P < 0.001, n = 13) (Figure 1B). Manually denuded arteries exhibiting a relaxation of <25% to 1 × 10−5 M acetylcholine when constricted with 1 × 10−5 M norepinephrine demonstrated an impaired PVAT anticontractile capacity, as demonstrated by an increase in contractility of arteries in response to increasing concentrations of norepinephrine (PKG+/− + PVAT + endothelium vs. PKG+/− + PVAT − endothelium; P < 0.001, n = 13) (Figure 1A) and in solution transfer experiments (Δ tension: PKG+/− + endothelium = 0.25 ± 0.034 mN/mm vs. PKG+/− − endothelium = 0.122 ± 0.027 mN/mm, P = 0.041, n = 13) (Figure 1B). The anticontractile effect induced by PVAT and the contribution of the endothelium were not due to a change in the capacity of arteries to contract, because KPSS-induced constriction between the groups was not significantly different (KPSS-induced constriction: no PVAT + endothelium = 0.282 ± 0.030 mN/mm; PVAT + endothelium = 0.342 ± 0.052 mN/mm; PVAT − endothelium = 0.247 ± 0.047 mN/mm, P = NS, n = 13).

Furthermore, the response was not due to a shift in sensitivity to norepinephrine, as ED50 values were not significantly different (data not shown).

3.2 PKG plays an important role in mediating the anticontractile capacity of PVAT

3.2.1 Evidence from myography dose–response experiments

Arteries with or without PVAT demonstrated no significant difference in the level of constriction to KPSS compared with PKG+/− littermates (PKG+/− − PVAT: 0.282 ± 0.030 mN/mm vs. PKG+/− − PVAT: 0.242 ± 0.052 mN/mm, P = NS, n = 13; PKG+/− + PVAT: 0.340 ± 0.052 mN/mm vs. PKG−/− + PVAT: 0.340 ± 0.039 mN/mm, P = NS, n = 13). PKG−/− mice demonstrated an increase in contractile response to cumulative concentrations of norepinephrine in both arteries with and without PVAT compared with PKG+/− littermates (PKG+/− vs. PKG−/−: no PVAT P = 0.004, n = 13; PVAT P < 0.0001, n = 13). Furthermore, PVAT from PKG−/− mice does not exhibit an anticontractile effect on the arteries, it surrounds because there was no significant...
Figure 1  Contractile responses of mesenteric arteries (A) PKG\(^{+/+}\) arteries to cumulative concentrations of norepinephrine (NE). PVAT exerts an anticontractile effect compared with arteries devoid of PVAT \((^{*}P < 0.05, n = 13)\), this effect is abrogated in the absence of the endothelium. (B) Solution transfer experiments demonstrating the transferable factor secreted by PVAT, transfer of bath solution between PVAT donor arteries to no PVAT recipient arteries causes a relaxation, which is reduced in the absence of the endothelium \((^{*}P < 0.05, n = 13)\). (C) PKG\(^{-/-}\) arteries with or without PVAT demonstrate similar contractile responses to cumulative concentrations of NE \((P = NS, n = 13)\). (D) Solution transfer experiments between PKG\(^{-/-}\) and PKG\(^{+/+}\) mice were demonstrating the role of PKG upstream and downstream of secreted PVAT factor(s). Donor (PVAT) or recipient (no PVAT) PKG\(^{-/-}\) arteries demonstrate impaired relaxation compared with control transfer experiments \((^{*}P < 0.05, n = 13)\). (E) Solution transfer experiments between PKG\(^{+/+}\) arteries in the presence of DT-2 or ODQ demonstrating incubation of recipient (no PVAT) arteries with ODQ and donor (PVAT) or recipient (no PVAT) arteries with DT-2 cause an abrogation of relaxation compared with control transfer experiments \(^{*}P < 0.05, n = 8\).
difference in arteries with or without PVAT from PKG\textsuperscript{\textminus/\textminus} mice in response to norepinephrine (\(P = 0.052, n = 13\)) (Figure 1C).

### 3.2.2 Evidence from myography solution transfer experiments

Solution transfer between PKG\textsuperscript{\textminus/\textminus} arteries with and without PVAT did not induce a significant change in tension in comparison with no PVAT to no PVAT control experiments (\(\Delta\) tension: 0.101 \(\pm\) 0.09 mN/mm, \(P =\) NS, \(n = 13\)). The involvement of PKG signalling in the anticontractile effect of PVAT was confirmed using the PKG\textsubscript{1\alpha} peptidic selective inhibitor DT-2 and sGC inhibitor, ODQ, which demonstrated that there was a significant increase in the contractile response to norepinephrine compared with PKG\textsuperscript{\textplus/\textplus} + PVAT arteries (PVAT vs. PVAT + DT-2: \(P = 0.025, n = 8\); PVAT vs. PVAT + ODQ: \(P = 0.049, n = 8\)).

Solution transfer experiments between pre-constricted PKG\textsuperscript{\textplus/\textplus} + PVAT donor arteries and PKG\textsuperscript{\textminus/\textminus} – PVAT recipient arteries were associated with a significantly impaired relaxation when compared with PKG\textsuperscript{\textplus/\textplus} + PVAT transfer (\(\Delta\) tension from pre-constriction: 0.153 \(\pm\) 0.040 mN/mm, \(P = 0.023, n = 13\)). Transfer of bath solution from PKG\textsuperscript{\textminus/\textminus} + PVAT donor arteries to PKG\textsuperscript{\textplus/\textplus} – PVAT recipient arteries was also associated with impaired relaxation (\(\Delta\) tension from pre-construction: 0.169 \(\pm\) 0.024 mN/mm, \(P = 0.043, n = 13\)) (Figure 1D). PKG\textsuperscript{\textplus/\textplus} arteries incubated with DT-2 in either recipient – PVAT arteries or donor + PVAT arteries caused a reduced relaxation in comparison with control (\(\Delta\) tension from pre-constriction: DT-2 – PVAT: 0.078 \(\pm\) 0.006 mN/mm, \(P = 0.010, n = 8\); DT-2 + PVAT: 0.152 \(\pm\) 0.019 mN/mm, \(P = 0.043, n = 8\)); however, ODQ reduced the change in tension when incubated in the without PVAT recipient arteries alone (\(\Delta\) tension from pre-construction: ODQ – PVAT: 0.086 \(\pm\) 0.016 mN/mm, \(P = 0.0168, n = 8\); ODQ + PVAT: 0.20 \(\pm\) 0.048 mN/mm, \(P = 0.1082, n = 8\)) (Figure 1E).

### 3.3 PKG activation has protective effects against experimental hypoxia

Induction of experimental hypoxia for 2.5 h caused a loss of the anticontractile effect in PKG\textsuperscript{\textplus/\textplus} animals, similar to previously published data\textsuperscript{16} (\(P = 0.016, n = 10\)), but had no effect in PKG\textsuperscript{\textminus/\textminus} arteries (\(P = 0.21, n = 10\)) (Figure 2), nor in vessels devoid of PVAT (\(P = 0.59, n = 10\)). The presence of ANP during experimental hypoxia was able to prevent the loss of the anticontractile capacity of PVAT in PKG\textsuperscript{\textplus/\textplus} arteries (\(P = 0.0093, n = 8\)) (Figure 2A) but had no effect in arteries from PKG\textsuperscript{\textminus/\textminus} mice (\(P = 0.11, n = 8\)) (Figure 2B) and was dependent on the presence of PVAT (data not shown).

### 3.4 Adiponectin/PGK interplay mediates normal PVAT function

#### 3.4.1 Adipo\textsuperscript{\textminus/\textminus} mice have impaired PVAT function

The contractile response of arteries from adipo\textsuperscript{\textminus/\textminus} mice was similar in the presence of PVAT to that of arteries devoid of PVAT, suggesting that the capacity of PVAT was compromised in adipo\textsuperscript{\textminus/\textminus} mice (PVAT vs. No PVAT: \(P = 0.1486, n = 6\)) (Figure 3A). Endothelium integrity was attenuated in adipo\textsuperscript{\textminus/\textminus} mice as demonstrated by a reduced ability to relax to acetylcholine (\(1 \times 10^{-5}\) mol/L) (\(P = 0.047, n = 6\)). As with PKG\textsuperscript{\textminus/\textminus} animals, experimental hypoxia had no significant effect on the contractile profile of arteries with or without PVAT from PKG\textsuperscript{\textplus/\textplus} mice (PVAT vs. PVAT + hypoxia: \(P = 0.6507, n = 6\)) (Figure 3B). Incubation with ANP was unable to restore the anticontractile property of PVAT (PVAT + hypoxia vs. PVAT + hypoxia + ANP: \(P = 0.1538, n = 6\)); furthermore, inhibition of PKG\textsubscript{1\alpha} with the selective peptidic inhibitor, DT-2, did not result in any further abrogation of PVAT function in adipo\textsuperscript{\textminus/\textminus} mice (\(P = 0.9618, n = 6\)) (Figure 3C).

#### 3.4.2 Immunohistochemical detection of adiponectin is reduced by hypoxia and increased by ANP

Immunohistochemical detection of adiponectin demonstrated staining around the adipocyte membranes of PVAT in PKG\textsuperscript{\textplus/\textplus} mice stimulated with norepinephrine (\(1 \times 10^{-5}\) mol/L) (Figure 4A). The expression of adiponectin as detected by immunohistochemistry was reduced following experimental hypoxia (Figure 4B) (normoxia: 7019 \(\pm\) 974.2 vs. hypoxia: 4137.4 \(\pm\) 849.3, \(P = 0.041\)), which was prevented by the inclusion of the PKG activator, ANP, during the period of hypoxia (Figure 4C) (4137.4 \(\pm\) 507.9, \(P = 0.049\)).

#### 3.4.3 Immunohistochemical detection of superoxide dismutase is increased in PKG\textsuperscript{\textminus/\textminus} mice independent of adipocyte size

The adipocyte surface area of PKG\textsuperscript{\textminus/\textminus} mice was not significantly different to PKG\textsuperscript{\textplus/\textplus} littermates (740.8 \(\pm\) 59.9 \(\mu\)m\(^2\) vs. 879.1 \(\pm\) 89.3 \(\mu\)m\(^2\)) respectively, \(P = 0.188\)) (see Supplementary material online). Significant differences in the intensity for SOD1 expression were observed, with
we focused particularly on the role of PKG in its production and activity.4,7 We have demonstrated that the absence of PKG is associated with a loss of the anticontractile capacity of PVAT, increased oxidative stress, and reduced adiponectin expression. Furthermore, hypoxia-induced loss of PVAT function is due to a reduction in adiponectin bioavailability and can be rescued by ANP via its effects on PKG. Solution transfer studies between control arteries and those from the PKG−/− mouse demonstrate that PKG is necessary for the release of adipokine-derived relaxing factor(s) including adiponectin from PVAT and the effects of these factor(s) on the smooth muscle and endothelium (Figure 6).

A number of recent studies have demonstrated the presence of nitric oxide signalling components, including PKG, within adipocytes themselves,19 suggesting that adipocytes may contribute to vascular relaxation in concert with the endothelium, as confirmed by our data in endothelium-denuded wild-type arteries in the presence of PVAT, where there is a significant reduction of the anticontractile effect in response to adrenergic stimulation. These data support our previous findings, where NOS inhibition inhibits PVAT function in human gluteal and mouse mesenteric arteries,4,9 as well as a role for endothelium-derived NOS in the PVAT effect observed in the rat aorta.3

Solution transfer studies from NE-pre-constricted donor (PVAT) arteries incubated with the PKG1α-selective inhibitor DT-2,18 to recipient (no PVAT) arteries pre-constricted with NE alone, were associated with a reduction in the change of tension. This effect did not occur when ODQ was used in donor arteries, suggesting that the specific inhibition of PKG is important in the PVAT-derived effect as opposed to simply the NO signalling pathway. Accumulating evidence indicates that stimulated adipocytes are able to produce nitric oxide.20–22 Furthermore, hypoxia has been demonstrated to attenuate cGMP effects in vascular smooth muscle cells13,24 and has been shown to change the redox regulation of sGC and downstream vascular responses25; therefore, the presence of PKG in the adipocyte is significant, particularly in relation to the anticontractile capacity of PVAT. Furthermore, data from our laboratory show that BKCa, a known phosphorylation target for PKG, mediates the PVAT effect; indeed, the hyperpolarization associated with adiponectin is inhibited by BKCa blockade.

The finding that PKG was involved in mediating the downstream effects of secreted adipokine(s) supports previous studies: NO has been implicated in the vascular response of a number of key adipokines including adiponectin,26,27 leptin (reviewed in Joffin et al.28), omentin,29 and resistin30; PKG plays a key role in the NO signalling pathway (reviewed in Francis et al.31), therefore, its absence is likely to disrupt the propagation of endothelium-mediated adipokine induced relaxation. However, taken together our data serve to highlight the importance that PKG inhibition has on normal vascular reactivity. Interestingly, eNOS, known to work upstream of PKG, is involved in the production of adiponectin in adipose tissue through its role in mediating mitochondrial function.32

We tested whether PKG has any significance in vascular contractility in an experimental model of oxidative stress by induction of experimental hypoxia.16 Our data show a loss of PVAT activity in PKG−/− mice, however, hypoxia had no significant effect on arteries from PKG−/− mice. This is due to increased levels of oxidative stress within the adipose environment, as oxidative stress has been associated previously with impaired PKG activation in response to cGMP.33 Interestingly, if arteries were incubated with ANP, a known activator of PKG,34 during the period of experimental hypoxia, there is a significantly reduced loss of PVAT function. It may be that ANP affects other molecules which may impact on the relaxation of the smooth muscle, for example, through

Figure 3 Contractile responses of mesenteric arteries from adipo−/− mice (n = 6). (A) Adipo−/− with or without PVAT demonstrate similar contractile responses to cumulative concentrations of NE. (B) Experimental hypoxia has no effect on the contractility of adipo−/− arteries with PVAT. (C) PKG activation or inhibition with ANP and DT-2, respectively, had no significant effect on adipo−/− arteries + PVAT.

4. Discussion

For the first time, we have demonstrated that PKG is important in mediating PVAT function both in regard to the secretion of adipose-derived relaxing factor(s) and in mediating their vasodilator activity. Because of our previous work which has identified adiponectin as one such adipokine,
Figure 4  Immunohistochemical detection of adiponectin in PVAT of PKG\(^{+/+}\) mice. (A) Expression of adiponectin following NE stimulation. (B) Adiponectin positive staining was reduced following experimental hypoxia. (C) Adiponectin staining of hypoxia + ANP PVAT was similar to NE stimulated samples. (D) IgG control.

Figure 5  Immunohistochemical detection of SOD1 in PVAT of PKG\(^{+/+}\) and PKG\(^{-/-}\) mice. (A) Expression of SOD1 in PKG\(^{+/+}\) mice (B) expression of SOD1 in PKG\(^{-/-}\) mice. (C) IgG control. (D) Intensity of staining from three separate images and mice was significantly higher in PKG\(^{-/-}\) compared with PKG\(^{+/+}\) mice \((P = 0.003)\).
cross-talk between cGMP and cAMP pathways; however, the absence of any beneficial effects in the PKG-/- mouse arteries and in the presence of PKG1 inhibitory DT-2 implicates PKG in mediating the protective effects of ANP in this experimental model.

Treatment of human primary adipocytes with ANP has been shown previously to increase the secretion and expression of adiponectin, one of the most abundant adipokines. Furthermore, incubation of human adipocytes with a PKG inhibitor reduces adiponectin secretion; therefore, we wished to determine whether the loss of PVAT function in PKG-/- mice was associated with impaired adiponectin bioavailability, which our previous studies have implicated as a key adipokine in the anticontractile capacity of PVAT. Expression of adiponectin was reduced in the adipose tissue of PKG-/- mice as determined by immunohistochemical detection of adiponectin, confirming our earlier findings that PKG plays a role in the secretion of adipokine(s) responsible for PVAT function, which we have identified as adiponectin. Using an adiponectin -/- mouse, we confirmed a loss of PVAT function and an impaired endothelial integrity. We believed that the loss of PVAT function was lost in these mice due to the lack of adiponectin and subsequent inability to activate NO, and that activation of PKG, through increased cGMP levels, would result in the restoration of signalling pathways downstream of adiponectin; however, when ANP was incubated with arteries from adiponectin -/- mice surprisingly, there was no restorative effect of the PKG signalling pathway and therefore no re-establishment of PVAT anticontractile function. Reviewing the available literature suggests that adiponectin -/- mice have a dysregulated NO signalling pathway and that the expression of the components of the pathway is reduced. This would support data from obese subjects in which adiponectin levels are reduced in obese subjects. However, the authors acknowledge that it is likely other adipokines and will be altered in the absence of PKG. Interestingly, recent data have shown an inverse relationship between PKG expression in mice and high-fat diet, furthermore, animals overexpressing PKG were protected from the detrimental effects of obesity induced by high-fat diet. Owing to the impaired gastrointestinal peristalsis associated with the PKG-/- mouse, it is difficult to investigate the importance of PKGI in the obese state; although a targeted knockout model would be perhaps useful to facilitate these investigations, our results clearly demonstrate that the expression of PKG goes beyond a single cell type of the vasculature. However, we accept it is necessary to investigate PKG signalling in the obese state to understand its importance in obesity.

Ultimately, the important finding of this study is that PKG plays a key role in regulating normal PVAT function and that PKG is necessary upstream of adiponectin expression and downstream of adiponectin

Figure 6 PKG mediates adiponectin bioavailability. The figure presents a proposed pathway for adiponectin release mediated by PKG1 signalling and how it is influenced by hypoxia. ANP-activated PKG1 may modulate the release of adiponectin by influencing mitochondrial function an effect inhibited by a PKG1 selective inhibitor (D) DT-2. Secreted adiponectin from the adipocyte subsequently activates adiponectin receptors (AdR) on the smooth muscle leading to downstream signalling events including AMPK activation, the phosphorylation of eNOS and ensuing nitric oxide (NO) signalling and endothelium-dependent relaxation. Activated PKG1 can then feedback to the mitochondrion to regulate adiponectin secretion and/or NO release at the endothelial level may stimulate soluble guanylate cyclase (sGC) in the adipocyte, which can be inhibited by 1H-(1,2,4)oxadiazolo(4,3-a)quinoxalin-1-one (ODQ). Hypoxia can inhibit eNOS and also changes the redox regulation of sGC and thereby influencing downstream vasomotor responses.
PKG and PVAT anticontractile effect

Supplementary material

Supplementary material is available at Cardiovascular Research online.

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