C25

Two apoptotic pathways are rapidly activated in response to oxidative stress in pancreatic acinar cells

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The pancreatic acinar cells are an excellent model to study intracellular Ca^{2+} signalling. We have shown previously that in normal pancreatic acinar cells (freshly isolated from humanely killed CD1 mice) the oxidant menadione evokes repetitive cytosolic Ca^{2+} spikes, partial mitochondrial depolarisation, cytochrome c release and apoptosis. The physiological agonists acetylcholine (ACh) and cholecystokinin also evoke cytosolic Ca^{2+} spikes, but neither depolarise mitochondria nor induce apoptosis. Ca^{2+} spikes induced by low ACh concentrations are confined to the apical secretory pole of the cell by the buffering action of perigranular mitochondria. Menadione prevents mitochondrial Ca^{2+} uptake, permitting rapid spread of Ca^{2+} throughout the cell and activation of caspase-9 and -3 (Gerasimenko et al. 2002). The Ca^{2+} chelator BAPTA prevents cytosolic Ca^{2+} spiking, blocks the menadione-elicited mitochondrial depolarisation and blocks menadione-induced apoptosis. Menadione induced apoptosis in this cell type is calcium dependent and rapid, with cytochrome c release occurring at 2 min. Our recent studies show real time activation and spatial distribution of caspase-9 and -3 using confocal microscopy of fluorescent caspase substrates. Here, we show conclusively that caspase-9 activation in response to menadione is rapid (the time to 1/2 max. activation ($t_{1/2}$) was 129 ± 43 s; n=12) and calcium dependent and that caspase-9 may be activated at or close to mitochondria in response to menadione in pancreatic acinar cells.

Gerasimenko J V et al. (2002). J Cell Sci 115, 485-497.

Where applicable, the experiments described here conform with Physiological Society ethical requirements.

C26

Inhibition of Shp-2 prevents bFGF-dependent endothelial cell proliferation and induces apoptosis

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Basic fibroblast growth factor (bFGF) has been shown to initiate signalling pathways important for cell proliferation and cell survival, both key steps in angiogenesis. The Src homology-2 domain containing tyrosine phosphatase (Shp-2) can be activated by bFGF, but its role in bFGF-dependent signalling is unknown.

Using antisense oligonucleotide (AS ODN) magnetofection, a technique where AS ODNs coupled to nanoparticles are rapidly delivered to cells under influence of an external magnetic field, we investigated the role of Shp-2 in cell proliferation and survival. All experiments were performed with human microvascular- and umbilical vein endothelial cells (HMEC, HUVEC). Data are presented as means ± S.E.M. Student's t test was used for analysis. Results were considered significant when $p<0,05$. AS-ODN magnetofection against Shp-2 led to a knock-down of Shp-2 protein (0.5µg AS-ODN/ml)in HUVECs and HMECs as assessed by Western blotting. Basal proliferation of HMECs, as measured by MTT reduction, was significantly inhibited up to 41% in Shp-2 AS ODN-treated cells as compared to nonsense oligonucleotides (NS ODN)-treated cells (± 5% $p<0.05$, n=12). In addition, bFGF (10ng/ml)-dependent proliferation following Shp-2 knock down was impeded to a similar extent (by 57 ± 13 %; $p<0.05$, n=12). To investigate if this decrease was due to an enhanced apoptosis, cell cycle analysis by flow cytometric propidium iodide and subsequent Annexin V staining were performed. This revealed a 1.4-fold increase in cells detected in the subG0 fraction (± 0.5; $p<0.05$, n=6) and a significant rise in Annexin V-positive cells (n=9) following Shp-2 AS ODN transfection, in contrast to NS ODN-treated cells. This increase of apoptosis was associated with a decreased phosphorylation of the PI3-kinase regulatory subunit p85 (24 ± 6%, $p<0.05$, n=3) and its downstream target Akt (n=4). A diminished phosphorylation of the MAP kinase ERK 42/44 was also observed despite bFGF stimulation (n=4).

Our results indicate that Shp-2 protects human endothelial cells from apoptosis, possibly by enabling bFGF-dependent activation of PI3-K and/or MAP kinase. Inhibition of Shp-2 increases apoptosis rates of endothelial cells which leads to an inhibition of basal and prevention of bFGF-dependent proliferation.

Where applicable, the experiments described here conform with Physiological Society ethical requirements.

C27

Role of PI3/Akt kinase in the infarct size-limiting effects of chronic hypoxia and ischaemic preconditioning

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Chronic intermittent high altitude (IHA) hypoxia leads to long-lasting adaptation protecting myocardium against all major manifestations of acute ischaemia/reperfusion (I/R) injury. Molecular mechanism of this phenomenon is less understood, and direct evidence is confined to some signalling elements utilized by ischaemic preconditioning (IP) including reactive oxygen species (ROS), protein kinase C and mitochondrial K(ATP) channels [mK(ATP)], whereas the role of pro-survival kinases remains elusive (1). Recent observations suggest that activation of PI3/Akt kinase promotes cardiomyocyte survival through a complex pathway that involves opening of mK(ATP) and generation of ROS. However, less is known about its effect on clinically relevant end-points, such as myocardial infarction.

In adult male Wistar rats, we examined the role of PI3/Akt in the infarct-limiting effect of long-term IHA hypoxia simulated in a

hypobaric chamber (7000 m, 8 h/day, 25 exposures) compared with short-term protection by IP. Both open-chest artificially ventilated animals (n=8-10 per group) and rats employed for the *ex vivo* studies (n=10-12 per group), were anaesthetised with sodium pentobarbitone (60 mg/kg b.w.). Data were expressed as means ± S.E.M. One-way ANOVA and Student-Newman-Keuls test were used for analysis of differences between the groups with $P<0.05$ considered as significant. Adaptation to hypoxia reduced the size of infarction (IS) induced by 20-min LAD coronary artery occlusion and 3-h reperfusion (TTC staining) from 64.9 ± 5.1% of the area at risk (AR) in the normoxic controls to 51.8 ± 4.4% in the IHA group ($P<0.05$). In the Langendorff-perfused hearts, IP induced by 2 episodes of ischaemia (5 min each) prior to a similar protocol of test I/R decreased IS/AR to 15.2 ± 1.0% as compared with 42.0 ± 5.1% in the controls ($P<0.05$). Enhanced Akt phosphorylation was observed in the adapted hearts. To explore the role of PI3/Akt in anti-infarct protection, PI3/Akt inhibitor LY294002 was given (0.3 mg/kg, i.v.) 5 min before I/R in the *in vivo* model. In the isolated hearts, LY (5μM) was administered in the perfusion medium 15 min before I/R or bracketing IP. The size of infarction was not affected by LY in the non-adapted controls in both models. In isolated hearts, LY completely abolished cardioprotective effect of IP and even enhanced the extent of myocardial injury (IS/AR 55.0 ± 4.2%) compared with controls. In the IHA rats, treatment with LY increased the size of infarction to that in the normoxic rats but a tendency to abrogate cardioprotection did not reach a level of significance (IS/AR 59.7± 4.1%).

In conclusion, the results suggest that activation of pro-survival PI3/Akt cascade plays a role in the infarct size-limiting mechanism and might represent a potential common protective pathway in the models of both, acute and chronic cardiac adaptation to oxygen deprivation.

Kolar F & Ostadal B (2004). Physiol Res 53(Suppl 1), S3-S13.

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Where applicable, the experiments described here conform with Physiological Society ethical requirements.

C28

Tissue injury and cytokines induce the expression of connexins and functional gap junctions in mouse dendritic cells

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Mature dendritic cells (DCs) establish homo- and hetero-cellular contacts that might favour the formation of gap junctions. We studied whether connexins (Cxs), protein subunits of gap junctions, are expressed by lymph node DCs in mice under normal conditions or after skeletal muscle damage. Double immunolabelling and confocal microscopy studies revealed frequent co-localization (82 ± 9%; n = 4 experiments) of Cx45 and DEC205 in lymph node DCs, whereas Cx43 was rarely found in these cells. However, Cx43 protein was strongly up-regulated in $DEC205^+$ DCs after skeletal muscle damage. Up-regulation of Cx43 gene expression by tissue damage was also demonstrated in mice carrying in one allele a β-galactosidase gene instead of the Cx43 coding region. In these mice numerous $DEC205^+$ DCs expressed β-galactosidase only after skeletal muscle damage ($Cx43^+/DEC205^+$ cells were 45.0 ± 3.5 cells/10^4 μm^2; n = 2 experiments). The effect of several mixtures or individual cytokines on the expression of functional gap junctions between DCs was tested in tsDCs, a dendritic cell line (1) as well as in bone marrow-derived DCs (BMDC) in primary culture. Animals were humanely killed for the primary cell culture for bone marrow. Under control conditions, tsDCs did not communicate via gap junctions as tested with the dye coupling technique (Lucifer yellow). However, after treatment with keratinocyte-conditioned medium (48 ± 3%; n = 3 experiments) or cytokine mixtures composed of TNF-alpha/IL-1beta or TNF-alpha/IL-1beta/IFN-gamma (38 ± 5% and 43 ± 5%, respectively; n = 3 experiments for each treatment), they became transiently coupled through a pathway sensitive to octanol, a gap junction blocker. Cellular coupling induced by both pro-inflammatory cytokine mixtures was prevented by IL-6. Single cytokines (TNF-alpha, IL-1beta, IFN-gamma or IL-6) or mixtures other than the described above did not induce coupling. Increased levels of protein and mRNA of Cx43 and Cx45 accompanied the appearance of cellular coupling. Similarly, BMDCs were not dye coupled under control conditions, but after treatment with TNF-alpha/IL-1beta they became transiently coupled. This response was abrogated in cells co-treated with IL-6. These studies provide the first demonstration of Cx expression and function in DCs. Previous studies have shown that formation of homotypic clusters of DCs induced by high cell density enhance T-cell stimulation, up-regulate co-stimulatory molecules, and the transfer of antigen between clustered DCs (2). Since clustered DCs might establish gap junctional communication, we propose that these membrane channels could mediate or coordinate functions of DCs as initiator of adaptive immunity.

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Where applicable, the experiments described here conform with Physiological Society ethical requirements.

PC121

The role of nicotinamide nucleotide transhydrogenase in insulin secretion and impaired glucose tolerance

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Insulin release from pancreatic β-cells is regulated by glucose metabolism. When plasma glucose levels rise, β-cell metabolism is stimulated causing intracellular ATP levels to increase, and thereby ATP-sensitive potassium (K_{ATP}) channels to close. This results in membrane depolarisation, activation of voltage-gated Ca^{2+} channels, influx of Ca^{2+} and exocytosis of insulin-containing vesicles. K_{ATP} channels in the β-cells of the inbred mouse strain, C57BL/6J, show impaired closure in response to glucose metabolism (1). However, they retain normal ATP sensitivity indicative of a defect further upstream perhaps at the level of β-cell metabolism (1). The glucose intolerance and reduced insulin secretion in these mice result in a phenotype reminiscent of human type-2 diabetes.

Through quantitative trait loci (QTL) mapping we identified nicotinamide nucleotide transhydrogenase (*Nnt*) as a strong candidate gene for the glucose intolerant phenotype in C57BL/6J mice. C57BL/6J mice have a multi-exon deletion in *Nnt* and >7-fold lower gene expression compared with controls. *Nnt* is a nuclear encoded mitochondrial gene catalysing the reversible reduction of $NADP^+$ by NADH.

To investigate the role of *Nnt* more closely, we used siRNA to knock down the expression of *Nnt* in the insulin-secreting cell line MIN6. Intracellular calcium and insulin secretion were measured in response to increasing extracellular glucose. Insulin secretion in response to external glucose (10mM) is substantially reduced (12.5±1.8ng/ml, *Nnt* siRNA (n=6) compared with 34.7±0.7ng/ml, nonsense siRNA (n=6)). Likewise, the glucose-dependent increase in $[Ca^{2+}]_i$ was dramatically decreased (87.9±13.6nM, *Nnt* siRNA (n=21) compared with 180.4±11.6nM, nonsense siRNA (n=18)).

We also identified two ENU-induced point mutations in the gene. These mutants were recovered as live mice by IVF, and the progeny were intercrossed to produce mice that are homozygous for the mutations. Intraperitoneal glucose tolerance tests (IPGTT), under local anaesthetic, at 12 and 16 weeks of age showed that both homozygous and heterozygous *Nnt* mutant mice are significantly glucose intolerant, in both males and females. They also have reduced insulin secretion in IPGTT.

In conclusion, a functional linkage between *Nnt* and both glucose intolerance and reduced insulin secretion was established. We hypothesise that *Nnt* knockdown impairs β-cell mitochondrial metabolism leading to less ATP production, and thereby lowered K_{ATP} channel activity. Consequently, glucose-dependent β-cell electrical activity and insulin secretion are impaired.

Toye AA et al. (2005). Diabetologia 48, 4.

Where applicable, the experiments described here conform with Physiological Society ethical requirements.

PC122

Protein tyrosine phosphatase activity in lymphoid cells under conditions of different models of ulcer development and ionizing irradiation treatment, combined with cycloferone application

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The levels of protein tyrosine phosphatase (PTP) activities in cytoplasmic membrane and cytosol of rat spleen and thymus lymphocytes under conditions of development of different models of stomach ulcer and whole body irradiation of rats at doses of 0.25-1 Gy were investigated. The effect of cycloferone administration on these parameters was evaluated.

Three models of stomach ulcer disease were used: aspirin-, stress- and ethanol-induced. It was shown that ethanol-induced stomach ulcer was accompanied by stimulation of PTP activities in membrane fractions of spleen and thymus lymphoid cells. A significant increase of thymocyte cytosol PTP activity and decrease of spleenocyte cytosol PTP activity were observed (Table 1).

Development of a stress-induced ulcer leads to thymocyte cytosol PTP activity stimulation and spleenocyte cytosol PTP activity inhibition. No changes were found in the activity of membrane PTP. Inhibition of PTP activities under conditions of aspirin-induced ulcer development was shown in most cases, except with thymus membrane-associated enzymes (Table 1). These data confirmed that different processes were involved in immune dysfunction accompanied ulcers with various aetiologies.

An increase of intracellular PTP activity and a decrease of membrane-associated activity was observed after addition of cycloferone, a synthetic interferon inducer (injections twice a day at a dose of 62 mg/kg for 5 days) (Table 1). Cycloferone application on rats with aspirin-induced ulcer leads to an increase in PTP activity in most cases (Table 1).

Cycloferone treatment before exposure to X-ray irradiation results in a shift of maximal increasing PTP activity levels to hither doses of irradiation in thymus and more potent activation of PTP activities in spleen (Table 1). Radioprotective features of the drug were confirmed also by cyclic nucleotide level measurement under the same conditions.

Table 1. Protein tyrosine phosphatase activity (nmol Pi / (min x mg)), in membrane fractions and cytosol of thymus and spleen lymphoid cells under conditions of stomach ulcer development and radiation treatment combined with cycloferone injections

	Sample			
	Thymus membrane fractions	Thymus cytosol	Spleen membrane fractions	Spleen cytosol
Control				
Without cycloferone	15.95±1.75	2.60±0.36	38.69±4.06	12.36±0.62
With cycloferone	7.38±0.91*	6.79±0.57*	7.63±1.06*	15.07±1.81
Ulcer				
Ethanol-induced without cycloferone	28.69±3.44*	12.35±2.03*	61.32±7.67*	3.91±0.58
Stress-induced without cycloferone	13.15±1.61	7.29±1.11*	36.01±4.21	6.01±0.88
Aspirin-induced without cycloferone	27.87±2.96*	0.79±0.22*	2.16±0.45*	0.32±0.04*
Aspirin-induced with cycloferone	74.80±6.74*	4.34±0.62*	15.72±2.06*	0.28±0.03
0.25 Gy irradiation				
Without cycloferone	12.72±1.39	3.39±0.31	38.7±3.2	18.16±2.17*
With cycloferone	11.07±1.38	6.28±0.62*	27.64±2.67*	29.05±4.06*
0.5 Gy irradiation				
Without cycloferone	21.44±2.41	6.49±0.52*	54.48±6.01*	10.24±1.12
With cycloferone	14.57±1.57	10.43±1.01*	33.8±4.05	14.69±1.54
0.75 Gy irradiation				
Without cycloferone	8.02±0.56*	5.07±0.71*	39.62±5.5	17.36±1.68*
With cycloferone	10.73±1.02*	15.20±1.82*	19.77±2.12*	8.69±1.04*
1.0 Gy irradiation				
Without cycloferone	8.36±1.25*	4.15±0.50*	33.16±4.97	6.46±0.79*
With cycloferone	19.77±2.56	13.11±1.36*	43.20±4.75	17.25±1.65*

Values are means±S.E.M.; n=6. *P≤0.5 compared with the control.

Where applicable, the experiments described here conform with Physiological Society ethical requirements.

PC123

NAADP, cADPR or IP_3 release Ca^{2+} from both the endoplasmic reticulum and an acidic store in the secretory granule area

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Inositol trisphosphate (IP_3) and cyclic ADP-ribose (cADPR) release Ca^{2+} from the endoplasmic reticulum (ER) via IP_3 and ryanodine receptors (RyRs), respectively. In contrast, nicotinic acid adenine dinucleotide phosphate (NAADP) may activate a novel Ca^{2+} channel in an acid compartment. We have used two photon permeabilized pancreatic acinar cells. Cells were isolated from CD1 male nouse pancreas (animals were humanely killed). We have shown that the three messengers tested could each release Ca^{2+} from the ER (n=25, 16 and 30, respectively) and also from an acid store in the granular region (n=10, 8 and 20, respectively). The NAADP action on both types of store, like that of cADPR but unlike IP_3, depended on operational RyRs, since it was blocked by high concentration of ryanodine (100 μM) or ruthenium red (10 μM) (n=5 and 9, respectively). In the whole cell the acidic store is approximately half of the size of the ER (47±11%, S.D., n=7). In the secretory granular area, the acidic store is 30±5% larger than the ER Ca^{2+} store (n=8). We estimate that the free $[Ca^{2+}]$ in the acidic store is 300±70 μM, whereas the free $[Ca^{2+}]$ in the ER is 120±50 μM (n=3). The acid Ca^{2+} store in the granular region did not have Golgi or lysosomal characteristics and is therefore most likely in the secretory granules. The ER is predominantly basal, but thin extensions penetrate into the granular area and Ca^{2+} signals probably initiate at sites where ER elements and granules come close together.

Where applicable, the experiments described here conform with Physiological Society ethical requirements.

PC124

Calcineurin and protein kinase C reciprocally regulate PAR-1-dependent Ca^{2+} release in human platelets

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An increase in intracellular calcium concentration ($[Ca^{2+}]_i$) is an important step in the activation of human platelets. Activation of protease-activated receptors (PARs) 1 and 4 by thrombin evokes release of Ca^{2+} from intracellular Ca^{2+} stores and Ca^{2+} influx, and also increases protein kinase C (PKC) activity in platelets. PKC regulates both Ca^{2+} entry (Rosado & Sage, 2000) and removal of Ca^{2+} from the cytosol. We have investigated the role of PKC in regulation of thrombin-evoked Ca^{2+} release.
$[Ca^{2+}]_i$ was recorded from fura-2-loaded platelets at 37°C. Statistical significance was analysed using a paired t test; $p<0.05$ was considered significant. Ro-31-8220 (5 μM, 5 min), which inhibits both conventional (Ca^{2+}-dependent) and novel (Ca^{2+}-independent) PKC isoforms, reduced the elevation in $[Ca^{2+}]_i$ evoked by thrombin (1 unit/ml) in the presence of 1 mM $CaCl_2$ to 67 ± 9 % of control (mean ± S.E.M.; n = 6; $p<0.05$). Ca^{2+} release (in the presence of 1 mM EGTA) was increased to 122 ± 7 % of control (n = 6; $p<0.05$), which may reflect regulation of the plasma membrane Ca^{2+} ATPase by PKC. The early spike in Ca^{2+} release, which has been attributed to activation of PAR-1 was abolished, indicating that PKC regulates thrombin-evoked Ca^{2+} release.
SFLLRN (30 μM), a PAR-1 agonist, stimulated Ca^{2+} release from intracellular Ca^{2+} stores. Ro-31-8220 reduced the maximum elevation of this release to 69.5 ± 4.0 % of control (n = 7; $p < 0.005$). In contrast, Gö6976, which selectively inhibits conventional PKC isoforms, had no significant effect on the Ca^{2+} release (n = 6), suggesting that activation of a novel PKC isoform may have a positive regulatory role in PAR-1-evoked Ca^{2+} release.
Cyclosporine A (CSA), which inhibits the type 2B serine/threonine phosphatase, calcineurin, is known to also induce platelet hyperaggegability. CSA (10 μM, 10 min) significantly enhanced the SFLLRN-evoked maximum elevation in $[Ca^{2+}]_i$ to 126.7 ± 5.6 % (n = 12; $p < 0.001$). However, CSA had no effect on the Ca^{2+} release in cells that had also been treated with Ro-31-8220 (n = 7), suggesting that Ca^{2+} release is reciprocally regulated by PKC and calcineurin.
In conclusion, PAR-1-dependent Ca^{2+} release is enhanced by PKC and inhibited by calcineurin. Calcineurin may modulate changes in $[Ca^{2+}]_i$, and thus platelet activation, by regulating the level of PKC-dependent phosphorylation of proteins involved in Ca^{2+} signalling. A possible candidate protein for this regulation is the inositol-1,4,5-trisphosphate receptor. This PKC-dependent increase in Ca^{2+} release may lead to an increased store-operated Ca^{2+} entry and enhanced platelet activation.

Rosado & Sage (2000). *J Physiol* **529**, 159-169.

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Where applicable, the experiments described here conform with Physiological Society ethical requirements.

PC125

Antagonism of transforming growth factorβ signalling modulates matrix metalloproteinase expression and arterial remodelling following vascular injury

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Elevated levels of transforming growth factor-β (TGF-β) within the vessel wall have been implicated in restenosis following balloon angioplasty (1). We have previously shown that migration of adventitial fibroblasts contributes to arterial remodelling and neointima formation elicited by vascular injury (2). Migration of vascular cells is partly regulated by matrix metalloproteinases (MMPs) and their tissue inhibitors (TIMPs), and a balance between MMP and TIMP expression contributes to arterial remodelling under physiological and pathological conditions (3). TGF-β1 is known to modulate MMP and TIMP activity thus may facilitate adventitial cell migration (4). The present study has utilised vascular gene transfer of smad7, an endogenous inhibitor of TGFβ signalling (5), to investigate whether antagonism of TGF-β1 alters luminal loss and adventitial cell migration following balloon injury in rat carotid arteries. Rats were anaesthetised with intraperitoneal administration of ketamine hydrochloride (72 mg/kg) and xylazine hydrochloride (5 mg/kg) before adenoviral vectors coordinating expression of nuclear β-galactosidase (β-gal) or smad7 were applied to the perivascular surface of left common carotid arteries. Balloon catheter mediated carotid artery injury was performed 4 days after gene transfer and animals humanely killed 3, 7 and 14 days later. Migration of adventitial fibroblasts was determinied by tracking β-gal labelled cells in histological sections. Vascular collagen content was assessed by picrosirius red staining and expression of smooth muscle α-actin, MMPs and TIMPs were determined by immunohistochemistry. Uninjured arteries only expressed adventitial β-gal positive cells; however, following balloon injury, β-gal positive cells were observed within the medial layer of vessels and contributed to the population of cells within the neointima at 7-14 days. Overexpression of smad7 with β-gal resulted in a significant reduction in the number of β-gal labelled cells in the neointima (14±4 vs 38±5%, mean±S.E.M., n=12, $p<0.01$, Student's t test), concomitant with reduced luminal loss (0.34±0.02 vs 0.27±0.02 mm^2, n=12, $p<0.01$) and decreased adventitial α-actin expression and collagen content (64±3 vs 51±6%, n=12, $p<0.01$) compared with controls. Levels of MMP-2 were decreased and TIMP-1 expression enhanced in arteries transfected with smad7 compared to those overexpressing β-gal only. These findings may partly explain the beneficial effects of antagonizing TGF-β1 signalling by smad7 overexpression on attenuating adventitial cell migration and arterial remodelling following vascular injury.

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Where applicable, the experiments described here conform with Physiological Society ethical requirements.