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RESEARCH PAPER

Differential regulation of Akt, caspases and MAP kinases underlies smooth muscle cell apoptosis during aortic remodelling in SHR treated with amlodipine

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Background and purpose: The regression of aortic hypertrophy is initiated by a transient wave of smooth muscle cell (SMC) apoptosis in spontaneously hypertensive rats (SHR) treated with antihypertensive drugs, although the molecular pathways remain unclear.
Experimental approach: Enzymes involved in apoptosis regulation were examined daily during onset aortic remodelling in SHR treated with amlodipine (20 mg kg^{-1} day^{-1}).
Key results: Significant reduction of aortic SMC number occurred by day 3 of amlodipine, reaching -13% at 28 days, followed by a significant regression of medial hypertrophy by day 5, reaching -13% at 28 days. ISOL-positive (apoptotic) SMC nuclei increased by 4.6-fold between days 2 and 4, in temporal correlation with the activation of caspase-8 (2.7-fold) at day 2 only, caspase-3 at days 3 and 4 (1.7-fold) and caspase-9 at day 3 only (3.1-fold). Akt phosphorylation, a pro-survival pathway, was reduced prior to apoptosis at day 1 (-52%) and until day 3. During the first 6 days of amlodipine treatment, significant reduction in phosphorylation of mitogen-activated protein (MAP) kinases was transient for p38 (-46% at day 3 only) but continuous for ERK1/2 after 3 days (-40%), and for JNK after 4 days ($>$-50%).
Conclusions and implications: Amlodipine inhibition of Akt occurred prior to and during SMC apoptosis induction, a process mediated by the early activation of caspase-8 followed by caspase-9 and -3 and associated with MAP kinase inhibition. These findings provide insights about the molecular pathways underlying SMC apoptosis leading to vascular remodelling during amlodipine treatment of hypertension.
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Keywords: hypertension; hypertrophy/hyperplasia; calcium channel blockers; apoptosis; smooth muscle cells

Abbreviations: AT_1, angiotensin II type 1 receptor; CSA, cross-sectional area; ERK1/2, extracellular signal-regulated kinase 1/2; FLIP, FLICE-inhibitory protein; ISOL, *in situ* oligonucleotide ligation; JNK, c-Jun N-terminal kinase; MAP, mitogen-activated protein; p38, p38 MAP kinase; SHR, spontaneously hypertensive rat; SMC, smooth muscle cells

Introduction

The importance of pleiotropic effects of anti-hypertensive drugs is increasingly being recognized (Mason *et al*., 2003; Nissen *et al*., 2004; Ichihara *et al*., 2006). Notably, vascular hypertrophy contributes to the pathogenesis of hypertension and the reversal of this pathological remodelling is suggested to increase the effects of anti-hypertensive drugs beyond the simple reduction of blood pressure (Intengan and Schiffrin, 2001). In conductance vessels of spontaneously hypertensive rats (SHR), a genetically determined model of hypertension, alterations in the replication and death rates of smooth muscle cells (SMC) contribute to establish the hypertrophy observed in adults (Thorin-Trescases *et al*., 2001). Pathological vascular remodelling associated with SMC hyperplasia is less readily reversible than remodelling associated with increased protein accumulation or cellular rearrangement around a smaller lumen. In recent years, SMC deletion by apoptosis has emerged as a mechanism by which vessels can control their mass in response to drugs or changes in haemodynamic influences (see deBlois *et al*., 2005; Gurbanov and Shiliang, 2006).

Studies showing an association between increased aortic SMC apoptosis and reversal of aortic hypertrophy in SHR treated with anti-hypertensive drugs shed a new light on the

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mechanisms of vascular remodelling (deBlois *et al*., 1997, 2005; Tea *et al*., 2000). A causal link between apoptosis and pharmacological reversal of vascular hypertrophy was suggested by the observation that only anti-hypertensive drug classes able to induce SMC apoptosis are also able to induce regression of hypertrophy in the aorta *in vivo* (deBlois *et al*., 1997). This hypothesis was later confirmed in SHR receiving a pan-caspase inhibitor in combination with the angiotensin II type 1 receptor (AT_1) antagonist losartan (Marchand *et al*., 2003). In this study, caspase inhibition prevented the early phase of hypertrophy regression in the aorta. The kinetics and mechanisms involved during initiation of SMC apoptosis with AT_1 receptor antagonists and with angiotensin converting enzyme inhibitors are becoming clearer (Tea *et al*., 2000; Marchand *et al*., 2003; Duguay *et al*., 2004).

However, the mechanisms by which calcium channel blockers regulate SMC *in vivo* remain largely undefined. Studies have shown that nifedipine induces SMC apoptosis *in vivo* (SHR) (deBlois *et al*., 1997) and *in vitro* in aortic cells derived from SHR (Stead *et al*., 2000) and that long-term treatment with amlodipine increases SMC susceptibility to apoptosis in the SHR aorta (Sharifi and Schiffrin, 1998). Moreover, amlodipine can induce apoptosis in leukaemia multidrug-resistant cells via a caspase-8-dependent pathway (Li *et al*., 2006a). In the current investigation, we sought to define the time course and molecular mechanisms of amlodipine-induced SMC apoptosis during aortic remodelling induced by amlodipine. Key enzymes regulating cell fate were examined, including the death-associated enzymes caspase-3, -8 and -9 (Lavrik *et al*., 2005), members of the mitogen-activated protein (MAP) kinases family (Pearson *et al*., 2001), the pro-survival serine-threonine kinase Akt and the lipid phosphatase phosphatase and tensin homologue (PTEN), involved in the metabolism of the Akt activator phosphatidylinositol (3,4,5) triphosphate (Datta *et al*., 1999). By implicating Akt inhibition and caspase-8 activation but not regulation of MAP kinases, as early events leading to SMC apoptosis, our current results provide insights about the molecular mechanisms underlying initiation of vascular remodelling during amlodipine treatment of hypertension.

Methods

Animal procedures

All animal procedures were conducted according to institutional guidelines. Male SHR ($n=116$) were purchased from Charles-River (St-Constant, Canada) and housed for 1 week before initiation of the protocol. Eleven-week-old SHR ($n=6$–20 per group) were killed after 0, 1, 2, 3, 4, 5, 6, 7, 8, 10, 14 and 28 days of treatment with the calcium channel blocker amlodipine (20 mg kg^{-1} per day) in the drinking water. Food and water were administered *ad libitum* and animals were maintained under a 12-h light/dark cycle. Body weight and water consumption were measured daily during the first week of treatment and three times a week for the remainder of the treatment, and the concentration of amlodipine in the drinking water was adjusted accordingly to ensure a constant dosage of amlodipine. Systolic blood pressure and heart rate were measured by tail-cuff plethysmography in a subset of conscious, restrained SHR throughout amlodipine treatment (at days 0, 1, 2, 4, 6, 8, 12 and 25). At the time of death, rats were anaesthetized with a single subcutaneous injection of ketamine (80 mg kg^{-1}), xylazine (4 mg kg^{-1}) and acepromazine (2 mg kg^{-1}). To evaluate DNA synthesis *in vivo*, rats were injected intravenously with [^{3}H]thymidine (0.5 mCi kg^{-1}) 1.5 h before death and an intravenous injection of $CdCl_2$ (100 mM) was used to induce diastolic cardiac arrest. The thoracic aorta was isolated, cleaned of blood and adventitial fat and weighed. A 3-mm long ring was cut between the third and fourth intercostal arteries. The aortic rings were then fixed in 4% paraformaldehyde, processed according to routine histological procedures, paraffin-embedded and used for morphometric measurements. The endothelium was removed from the rest of the vessel, starting immediately after the aortic arch. The denuded vessel was immediately frozen in liquid nitrogen and later pulverized in liquid nitrogen using a mortar and pestle and stored at −80°C until analysis.

Hypertrophy and cell number measurements

The aortic medial cross-sectional area (CSA) was evaluated in 3 μm-thick, haematoxylin–phloxin–safran (HPS)-stained sections of aorta. Photomicrographs were taken at $\times 40$ magnification, digitized and analysed using the NIH Image 1.62 program (developed at the National Institute of Health; http://rsb.info.nih.gov/nih-image/). The three-dimensional disector method was used to measure SMC number per unit of length. Photomicrographs of three consecutive 3-μm-thick HPS-stained aortic sections were taken at $\times 400$ magnification and analysed as described previously (deBlois *et al*., 1997). In a subset of rats killed at days 0, 7 and 14, one aliquot of the powdered aorta was weighed and total tissue DNA was extracted by the phenol and chloroform procedure, following digestion steps with proteinase K and RNase A in the presence of ethylenediaminetetraacetic acid (Teiger *et al*., 1996). DNA concentration (micrograms of DNA per millimeter of aorta) was determined by spectrophotometry. DNA content per milligram of extracted tissue was multiplied by the weight of the aorta and then normalized for the length of the isolated aorta. *In vivo* DNA synthesis in the aorta was quantified by evaluating [^{3}H]thymidine incorporated into DNA 1.5 h before diastolic cardiac arrest, using the extracted DNA.

Detection of apoptosis

The degree of internucleosomal DNA fragmentation in the aortic media was quantified by radiolabelling DNA on free 3′-OH ends using terminal deoxynucleotidyl transferase and [^{32}P]-dCTP, as described previously (Duguay *et al*., 2004). The slope of the linear regression of radioactivity associated with 150–1500 bp DNA fragments plotted against the amount of DNA loaded on the gel (0.05, 0.1, 0.2 and 0.4 μg) was defined as the DNA fragmentation index (arbitrary units per microgram DNA). To get a more precise evaluation of the number of apoptotic cells, SMC nuclei showing evidence of internucleosomal DNA fragmentation were detected using the *in situ* oligonucleotide ligation (ISOL) assay as described by

the manufacturer, with minor modifications. Briefly, 5 μm sections of paraffin-embedded aorta were deparaffinized and rehydrated, followed by a pretreatment with proteinase K (50 μg ml^{-1}). Sections were then incubated with the equilibration buffer before ligation of the biotinylated oligonucleotide A with T4 DNA ligase for 19 h at 18°C. Sections were then incubated with streptavidin-Alexa Fluor 488 (25 μg ml^{-1}) and propidium iodide (5 nmol l^{-1}) was used to stain all nuclei with red fluorescence. Slides for positive controls were pretreated with DNAse 1 (200 μg ml^{-1}, 10 min) and negative controls had water instead of DNA ligase. Every ISOL-positive SMC nuclei (in green) per cross-section was counted using fluorescence microscopy. As described by Didenko *et al*. (1999), this method labels only DNA presenting double-stranded breaks with single-base 3′ overhangs and is thus more specific to apoptosis than the transferase (TdT)-mediated dUTP nick-end-labeling assay.

Immunoblots

Proteins in rats killed between days 0 and 6 were extracted from the powdered aorta and were used for western blot analysis as described previously (Der Sarkissian *et al*., 2003), with minor modifications. Briefly, aliquots containing 25 μg of extracted aortic proteins were loaded onto 10 or 15% sodium dodecyl sulphate-polyacrylamide gel and transferred to Hybond-C membrane after electrophoresis. For caspase expression analysis, antibodies recognizing both the cleaved and the pro-form of the enzymes were used. Membranes were hybridized with antibodies directed against caspase-3 (235412; Calbiochem, San Diego, CA, USA, 1:2000), caspase-8 (D-8; Santa Cruz, Santa Cruz, CA, USA, 1:1000) or caspase-9 (9506; Cell Signaling, Danvers, MA, USA, 1:500), followed by incubation with the appropriate horseradish peroxidase-conjugated secondary antibody (Jackson, West Grove, PA, USA). Membranes were then incubated with enhanced chemiluminescence (ECL) Plus, exposed to film and developed. A similar procedure was used to evaluate Akt phosphorylation (4051; Cell Signaling, 1:4000), total Akt (9272; Cell Signaling, 1:5000), PTEN phosphorylation (9554; Cell Signaling, 1:1000), total PTEN (9556; Cell Signaling, 1:2000), extracellular signal-regulated kinase 1/2 (ERK1/2) phosphorylation (9101; Cell Signaling, 1:4000), total ERK1/2 (9102; Cell Signaling, 1:1000), p38 MAP kinase (p38) phosphorylation (4631; Cell Signaling, 1:2000), total p38 (9212; Cell Signaling, 1:1000), c-Jun N-terminal kinase (JNK) phosphorylation (9251; Cell Signaling, 1:500) and total JNK (9252; Cell Signaling, 1:3000).

Statistical analysis

Analysis of variance followed by Dunnett's multiple comparisons test was applied for all data. Values are presented as mean ± s.e.m. and $P<0.05$ was considered statistically significant.

Drugs, chemical reagents and other materials

Amlodipine was a gift from Pfizer Canada. Ketamine was from Bioniche Animal Health Canada (Ontario, Canada), xylazine was from Bayer (Ontario, Canada) and acepromazine was from Ayerst (Ontario, Canada). [^{3}H]thymidine and propidium iodide were from MP Biomedicals (Solon, OH, USA). The ISOL kit was purchased from Chemicon (Temecula, CA, USA) and streptavidin-Alexa Fluor 488 was from Invitrogen (Ontario, Canada). [^{32}P]-dCTP, Hybond-C membrane and ECL Plus were purchased from GE Healthcare Bio-Sciences (Quebec, Canada). All other chemicals were purchased from Sigma-Aldrich, Oakville, ON, Canada.

Results

Pretreatment body weight was 276 ± 4 g and after a small decrease (−6%) at day 1, it increased throughout treatment to reach 308 ± 12 g at day 28. Water intake dropped at day 1 (−45%), returned to basal value at day 2 and remained unchanged afterwards. All rats were hypertensive before initiation of therapy, with a systolic blood pressure of 185 ± 2 mm Hg ($n=24$; Table 1). A reduction of 42 ± 5 mm Hg was observed at day 1 and systolic blood pressure stabilized at 148 ± 2 mm Hg after 25 days. Heart rate was significantly increased at day 2 only (409 ± 11 compared to 371 ± 6 beats per minute at day 0, $P<0.01$).

Aortic CSA, indicative of vessel hypertrophy, was rapidly reduced by amlodipine, reaching significance at day 5 (Figure 1a). During the 28 days of treatment, the greatest reduction of the aortic hypertrophy occurred between days 0 and 5 (−8.2 ± 2.4%), with minor modifications occurring between days 5 and 28 (cumulative reduction of 12.9 ± 2.7%).

As a first evaluation of cell population dynamics during the experimental period, DNA content, synthesis and fragmentation in the aorta were evaluated in a subset of SHR at days 0, 7 and 14 of amlodipine treatment. The reduction of aortic DNA content (−34 ± 11%, $P<0.05$ vs day 0) and DNA synthesis (−52 ± 6%, $P<0.01$ vs day 0) were maximal at day 7, while DNA fragmentation, indicative of

Table 1 Systolic blood pressure measured by tail-cuff plethysmography during treatment with amlodipine

	Days of amlodipine treatment							
	0	*1*	*2*	*4*	*6*	*8*	*12*	*25*
	(n = 24)	*(n = 6)*	*(n = 12)*	*(n = 20)*	*(n = 12)*	*(n = 12)*	*(n = 10)*	*(n = 6)*
Systolic blood pressure (mm Hg)	185 ± 2	143 ± 5*	136 ± 3*	142 ± 3*	139 ± 2*	138 ± 2*	149 ± 2*	148 ± 2*

Abbreviation: SHR, spontaneously hypertensive rat.
Values are mean ± s.e.m.
*Significantly different ($P<0.05$) from untreated SHR (day 0).

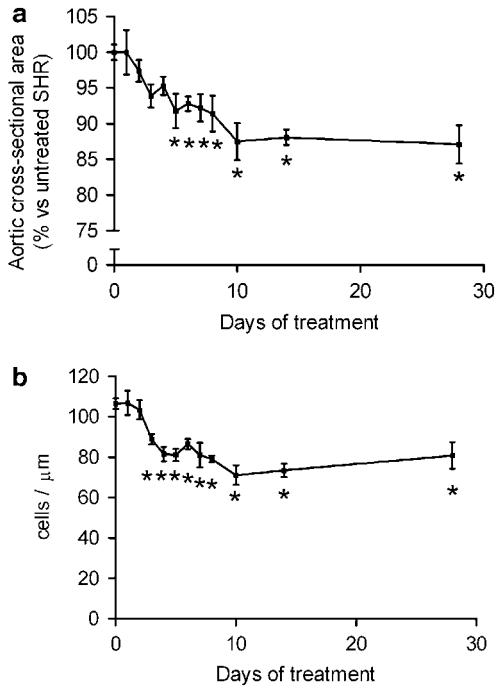

Figure 1 Time course of structural changes in SHR aorta after treatment with amlodipine for up to 28 days. In (**a**) the reduction in aortic CSA was significant after 5 days of treatment with amlodipine, whereas in (**b**) the reduction in aortic SMC number was significant after only 3 days of treatment with amlodipine. *Significantly different ($P<0.05$) from day 0. $n=20$ (day 0), $n=8$ (days 1, 7 and 14), $n=14$ (day 2), $n=10$ (day 3), $n=12$ (day 4 and 6) and $n=6$ (days 5, 8, 10 and 28).

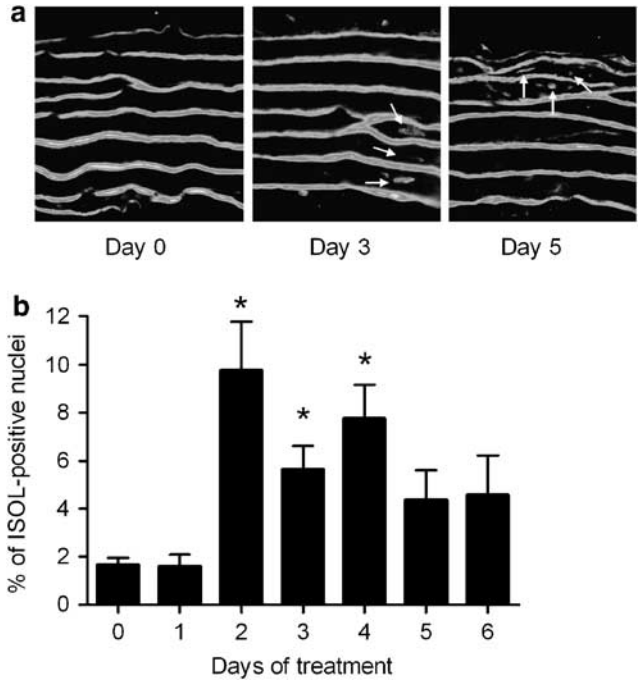

Figure 2 (**a**) Representative photomicrographs showing the increased number of aortic ISOL-positive (apoptotic) complete nuclei at day 3 and smaller fragments at day 5. (**b**) Bar graph of all ISOL-positive nuclei present on aortic sections of rats killed during 1 week of treatment with amlodipine. *Significantly different ($P<0.05$) from day 0. $n=7$ (days 0 and 2), $n=6$ (days 1, 3 and 5) and $n=8$ (days 4 and 6).

Table 2 Aortic DNA content, synthesis and fragmentation of SHR treated with amlodipine

	Days of amlodipine treatment		
	0	*7*	*14*
	(n=4)	*(n=4)*	*(n=4)*
DNA content ($\mu g\,mm^{-1}$)	1.31±0.09	0.86±0.15*	0.92±0.11*
DNA synthesis (CPM 100 $\mu g\,DNA^{-1}$)	500±40	240±31*	215±44*
DNA fragmentation index (arbitrary units $\mu g\,DNA^{-1}$)	4.77±0.92	8.06±.86*	9.45±1.31*

Abbreviation: SHR, spontaneously hypertensive rat.
Values are mean±s.e.m.
*Significantly different ($P<0.05$) from untreated SHR (day 0).

apoptosis, was significantly increased at days 7 and 14 (Table 2). Change in cell number was evaluated by the three-dimensional disector method to confirm and better define the kinetics of aortic SMC deletion with amlodipine. In agreement with the DNA content values, cell number in the aortic media was reduced within the first week of amlodipine treatment (Figure 1b). The most drastic deletion of SMC nuclei occurred between days 2 (−3.4±4.7% vs day 0) and 4 (−18.5±3.5% vs day 0).

Since the structural changes initiated by amlodipine mostly occurred during the first week of treatment, apoptosis induction and the regulation of mediators of cell death/cell growth was studied during this period. SMC nuclei with fragmented DNA, a hallmark of apoptosis, were detected *in situ* in the aortic media by the ISOL method. This technique specifically labels nuclei with double-stranded DNA breaks presenting single-base 3′ overhangs that are generated only during DNA fragmentation associated with apoptosis. Amlodipine induced a transient increase of ISOL-positive nuclei in the aorta, in temporal correlation with the kinetics of cell number reduction, with a significant peak between days 2 and 4 (Figure 2). Next, the activation of apoptosis regulators was studied in the aorta of SHR treated with amlodipine. Expression and activation of caspases were evaluated with antibodies detecting both pro- and cleaved forms of the enzymes. Initiator caspase-8 and -9 were significantly activated by amlodipine at days 2 and 3, respectively (Figures 3a and b). The ratio of cleaved caspase-8 over its pro-form increased by 2.7-fold at day 2 only ($P<0.01$ vs day 0), while the ratio of cleaved caspase-9 over its pro-form increased by 3.1-fold at day 3 only ($P<0.05$ vs day 0). The ratio of the cleaved form of the effector caspase-3 over its pro-form was significantly increased by amlodipine at days 3 and 4 (1.7-fold, $P<0.05$ vs day 0, Figure 3c). Cleaved caspase-3 and -9 were also normalized against β-actin and this analysis showed results (not shown) similar to the ratios of cleaved to pro-form caspase.

We then analysed the regulation of Akt, one of the most important survival signals in SMC. Akt phosphorylation (ratio over total Akt) was significantly and transiently

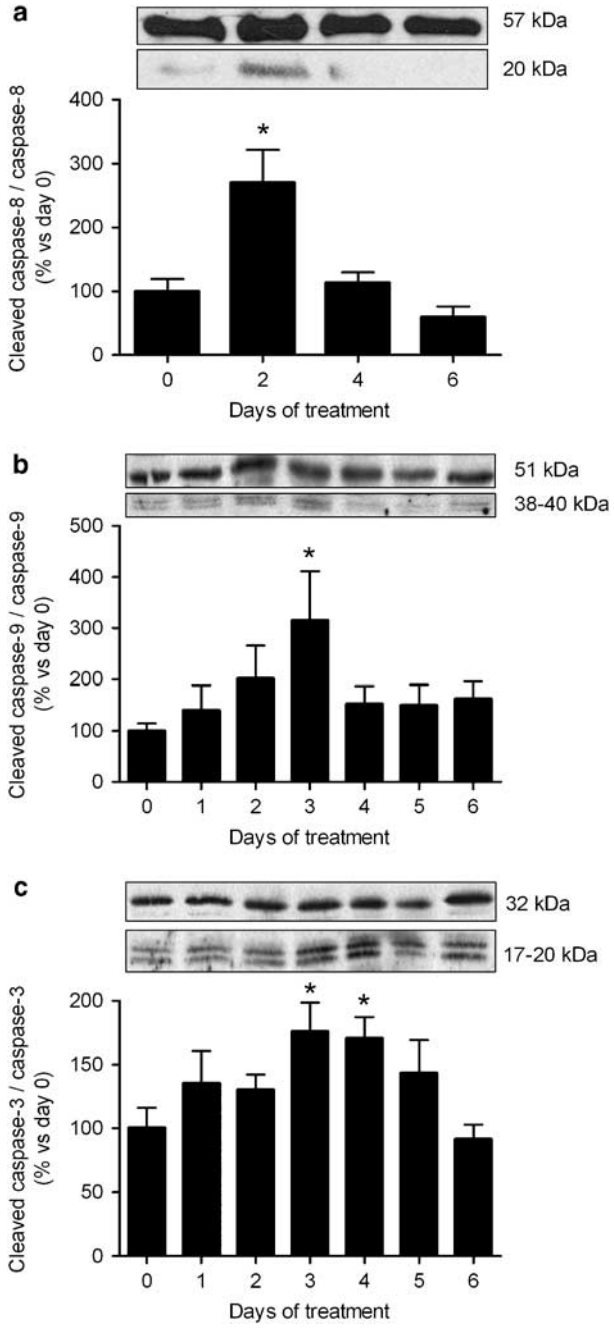

Figure 3 Bar graphs showing the aortic expression of cleaved caspases in SHR aorta in response to amlodipine. (**a**) Cleaved caspase-8 was increased at day 2 only. (**b**) Cleaved caspase-9 was increased at day 3 only. (**c**) Cleaved caspase-3 was increased at days 3 and 4. *Significantly different ($P<0.05$) from day 0. Insets show the representative expression of the cleaved form of each caspases studied. $n=6$ per group for caspase-8. $n=11$ (days 0, 1, 4 and 6), $n=12$ (day 2), $n=10$ (day 3) and $n=6$ (day 5) for caspase-3 and -9.

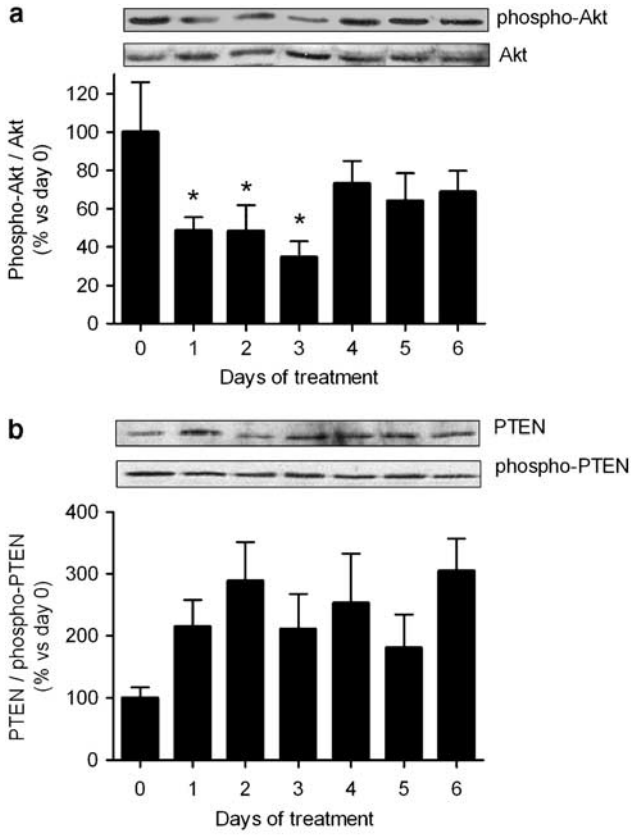

Figure 4 Bar graphs showing (**a**) the reduced phosphorylation level of Akt from days 1 to 3 and (**b**) the trend toward an increased PTEN/phospho-PTEN expression ratio from days 1 to 6. *Significantly different ($P<0.05$) from day 0. Insets show the representative expression of the phosphorylated form and the total form of both Akt and PTEN. $n=6$ (days 0, 4, 5 and 6), $n=9$ (days 1 and 3) and $n=7$ (day 2) for both Akt and PTEN.

reduced for 3 days only starting at 1 day after initiation of treatment with amlodipine ($-51.5\pm7.1\%$ vs day 0, $P<0.01$, Figure 4a). To examine whether activation of PTEN is implicated in the inhibition of Akt activation, the ratio of total PTEN (including the non-phosphorylated active form) over phosphorylated (inactive) PTEN was measured. Treatment resulted in a trend toward an increase in response, although the change did not reach statistical significance (Figure 4b). Members from the three families of MAP kinases showed differential regulation during the period of SMC apoptosis induction. p38 phosphorylation (ratio over total p38) was reduced by amlodipine only when apoptosis of SMC peaked, at day 3 ($-45.8\pm8.8\%$, $P<0.01$, Figure 5a). In contrast, ERK1/2 phosphorylation was significantly reduced in a sustained manner starting at day 3 (overall inhibition: $39.8\pm10.8\%$ vs day 0, Figure 5b) while JNK phosphorylation was significantly reduced in a sustained manner starting at day 4 (overall inhibition: $65.3\pm10.7\%$ vs day 0, Figure 5c).

Discussion

Despite their extensive use in treating cardiovascular diseases for more than two decades, mechanisms of vascular hypertrophy regression with calcium channel blockers remain incompletely defined and may include pleiotropic effects beyond the inhibition of SMC contraction (Mason *et al.*, 2003; Nissen *et al.*, 2004). On the basis of multiple criteria, we showed that amlodipine could induce therapeu-

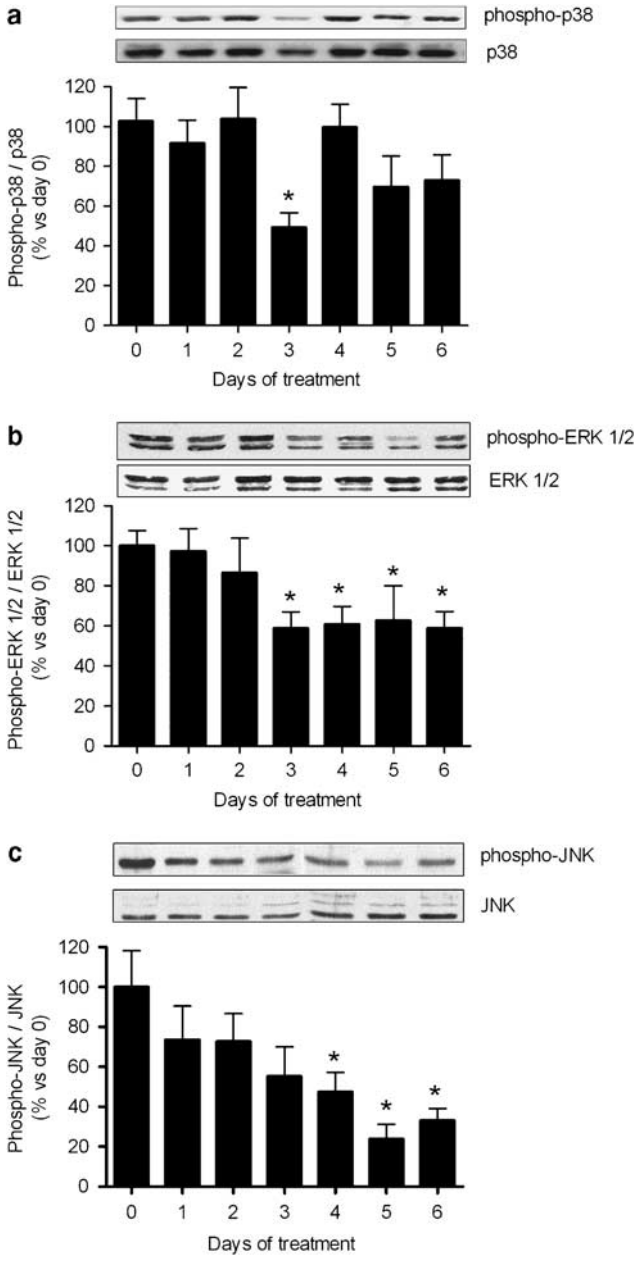

Figure 5 Bar graphs showing (**a**) the reduced phosphorylation level of p38 at day 3 only and (**b** and **c**) the reduced phosphorylation level of ERK1/2 from days 3 to 6 and of JNK from days 4 to 6. *Significantly different ($P<0.05$) from day 0. Insets show the representative expression of the phosphorylated form and the total form of each protein. For p38, $n=14$ (days 0, 1 and 4), $n=15$ (day 2), 13 (days 3 and 6) and $n=8$ (day 5). For ERK1/2 and JNK, $n=12$ (day 0), $n=11$ (days 1, 2 and 6), $n=9$ (days 3 and 4) and $n=6$ (day 5).

tic vascular SMC apoptosis *in vivo*. This effect lead to the normalization of SMC number down to levels seen in normotensive rats (Duguay *et al.*, 2004). The high-resolution time course revealed that SMC apoptosis was rapid and massive (~25% of cell deletion at 5 days). A key observation is that SMC apoptosis closely preceded the significant regression of aortic hypertrophy. The earliest event recorded was the reduction of Akt phosphorylation from days 1 to 3, before caspase activation, associating apoptosis induction with reduced survival signals. The significant reduction in Akt phosphorylation was accompanied by a nonsignificant trend toward activation of the lipid phosphatase PTEN, suggesting that alternative mechanisms may also contribute (for example, reduced phosphatidylinositol-3 kinase activity). Our results suggest that caspase-8 was the sole death enzyme activated at day 2, suggesting that apoptosis was triggered via the extrinsic death receptor pathway (Jin and El-Deiry, 2005). Consistent with these findings, amlodipine can induce apoptosis in leukaemia multidrug-resistant HL-60 cells (although at high concentrations) by a caspase-8-dependent mechanism (Li *et al.*, 2006a). Moreover, angiotensin II-induced apoptosis of non-quiescent SMC is mediated by reduced Akt phosphorylation and caspase-8 activation (Li *et al.*, 2006b). A growing body of evidence suggest that inhibition of Akt decreases expression of the caspase-8 homologue, FLICE-inhibitory protein (FLIP), notably in human tumour cells (Panka *et al.*, 2001) and endothelial cells (Skurk *et al.*, 2004). FLIP, which functions as a caspase-8-dominant negative, is expressed in arterial SMC where it can inhibit death receptor-induced apoptosis (Imanishi *et al.*, 2000; Wang *et al.*, 2002). Thus, the downregulation of FLIP following Akt inhibition may be permissive for caspase-8 activation. Suppression of Akt signalling induces Fas ligand expression and stimulates caspase activity and apoptosis in cultured SMC (Suhara *et al.*, 2002). This effect is dependent on JNK signalling, consistent with the proapoptotic influence of this MAP kinase pathway in most systems (Lin, 2003). In the present study, it is noteworthy that JNK activity did not decrease until after the transient wave of SMC apoptosis. Thus, the Fas receptor is a candidate pathway for caspase-8 activation and SMC apoptosis in our model. The late activation of caspase-9 and caspase-3 suggests caspase-8-mediated recruitment of the intrinsic mitochondrial pathway, possibly at the level of Bid cleavage by caspase-8 (Luo *et al.*, 1998) and leading to amplification of the death signal. It is intriguing to speculate that the downregulation of proapoptotic JNK signalling, along with the restoration of pro-survival Akt signalling, played a role in the termination of SMC apoptosis around days 4–5 of amlodipine treatment. These candidate mechanisms of SMC apoptosis initiation and termination with amlodipine are illustrated in Figure 6.

A significant finding of our time-course study is that reduced signalling via ERK1/2 and p38 occurred after, and therefore was not involved in, apoptosis initiation. SMC growth is dependent on activation of both the ERK1/2 and p38 (Touyz and Schiffrin, 2000). ERK1/2 and p38 activities are increased in the aorta of SHR (Kubo *et al.*, 2002; Umemoto *et al.*, 2006) and these are reduced by long-term treatment with amlodipine (Umemoto *et al.*, 2006). The fact that p38 was not inhibited in a sustained manner by amlodipine is probably due to the short treatment period (6 days). The transient inhibition of p38 synchronized with peak apoptosis activity is intriguing and may be related to the massive production of apoptotic bodies. Apoptotic bodies have been shown to modulate MAP kinases activity in macrophages, where they cause the inhibition of ERK1/2 and activation of p38 and JNK (Reddy *et al.*, 2002; Patel *et al.*,

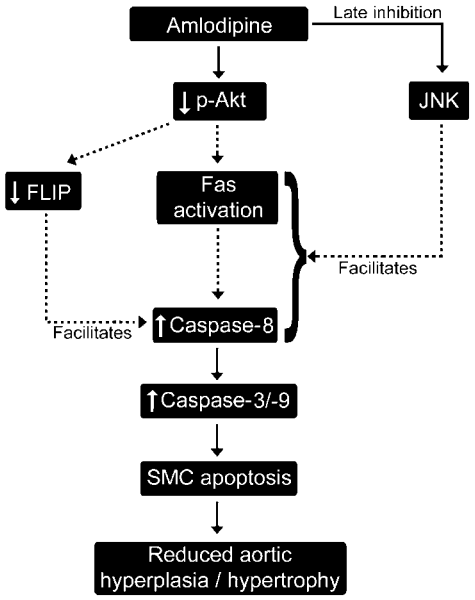

Figure 6 Scheme of a working hypothesis for apoptosis of aortic SMC in SHR, induced by amlodipine. The data suggest that amlodipine inhibition of Akt phosphorylation leads to the early activation of caspase-8 followed by caspase-9 and -3 activation. On the basis of the findings in other systems (see Discussion), putative pathways for apoptosis induction following attenuation of Akt include activation of the pro-death receptor Fas and downregulation of the caspase-8 inhibitor FLIP in the arterial wall. Sustained elevation of JNK early during amlodipine treatment may contribute to Fas signalling and death. Late inhibition of JNK and restoration of Akt signalling may contribute to the termination of SMC apoptosis.

2006). Whether these effects are cell-type specific is unknown.

As the regression of aortic hypertrophy reached significance after the massive deletion of cells, we propose that apoptosis played a causative role in this reversal of hypertrophy. This proposal is compatible with our earlier finding that co-administration of a pan-caspase inhibitor with losartan prevented both apoptosis induction and aortic mass regression (Marchand *et al*., 2003). Amlodipine also reduced *in vivo* DNA synthesis in a sustained manner, further contributing to a reduction of cell number by blunting proliferation. Possible mechanisms of growth inhibition include the modulation of SMC phenotype (Lai *et al*., 2002; Umemoto *et al*., 2006), the inhibition of growth factors (Lai *et al*., 2002) or MAP kinases activity (Zhang *et al*., 2000; Yin *et al*., 2005), and the activation of growth suppressors such as p21/WAF1 (Ziesche *et al*., 2004). The transient nature of apoptosis was highlighted by the short duration of caspases activation and the absence of further cell number reduction after the initial peak. Despite this, DNA fragmentation remained increased after the termination of caspase activation, possibly due to remaining apoptotic bodies as evidenced by small structures showing DNA fragmentation at day 5 (Figure 2a), that is more than 48 h after the termination of the massive apoptosis. Remnant DNA fragmentation and increased ratios of Bax:Bcl-2 protein expression have been observed in the SHR aorta after 12 weeks of amlodipine treatment (Sharifi and Schiffrin, 1998). Interestingly, a recent study showed that in normal (but not atherosclerotic) mouse arteries undergoing massive SMC apoptosis in response to diphtheria toxin, surviving SMC can effectively clear apoptotic bodies without significant inflammatory cell infiltration (Clarke *et al*., 2006). The same study revealed that contractile responses to receptor-dependent and -independent agonists were unaffected in vessels with more than 50% SMC deletion after 4 weeks of diphtheria toxin administration (Clarke *et al*., 2006). These data suggest that, in the long term, arteries can compensate for SMC loss, possibly through the development of cellular hypertrophy. In the short term, SMC deletion may reduce passive and active tension in arteries undergoing remodelling. The SHR model is remarkable in that apoptosis of up to 30% of SMC is induced in response to drugs that are routinely used in the clinic (deBlois *et al*., 1997). The rapidity and extent of SMC deletion in amlodipine-treated rats are reminiscent of what is seen in losartan-treated rats and in enalapril-treated rats (deBlois *et al*., 1997; Marchand *et al*., 2003 Duguay *et al*., unpublished observations), in which 30% SMC deletion also occurs within a time window of less than 2–3 days. Thus, to our knowledge this model shows one of the largest synchronized inductions of cell death by apoptosis *in vivo*. This feature is possibly related to the enhanced rates of SMC growth and polyploidy development in SHR (reviewed in deBlois *et al*., 2005). Consistent with this, SMC engaged in the cell cycle show an increased susceptibility to apoptosis induction (Bennett *et al*., 1994).

As a limitation to the present study, we cannot exclude pressure-dependent mechanisms to explain the effects of amlodipine, particularly the sustained changes (inhibition of ERK1/2 and JNK and DNA synthesis). It is unlikely, however, that the transient modulations were secondary to blood pressure reduction, including the inhibition of Akt and p38 and the induction of caspase-dependent cell death. Moreover, we previously reported that a similar reduction in blood pressure with hydralazine did not result in SMC apoptosis nor correction of vascular hypertrophy or hyperplasia in SHR (deBlois *et al*., 1997).

In summary, we report that onset of aortic hypertrophy regression with amlodipine in SHR immediately followed the loss of ~25% of medial SMC via apoptosis. Mechanisms of SMC death induction by amlodipine include the reduction of Akt-associated survival signalling and the activation of the extrinsic caspase-8-associated apoptotic pathway. If confirmed in humans, these conclusions may help to develop better interventions to control vascular structure and reduce complications associated with hypertension.

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Conflict of interest

The authors state no conflict of interest.

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