Cks1 Mediates Vascular Smooth Muscle Cell Polyploidization*

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Vascular smooth muscle cells (VSMC) at capacitance arteries of hypertensive individuals and animals undergo dramatic polyploidization that contributes toward their hypertrophic phenotype. We report here the identification of a defective mitotic spindle cell cycle checkpoint in VSMC isolated from capacitance arteries of pre-hypertensive rats. These cells demonstrated a high predisposition to polyploidization in culture and failed to maintain cyclin B protein levels in response to colcemid, a mitotic inhibitor. Furthermore, this altered mitotic spindle checkpoint status was associated with the overexpression of Cks1, a Cdc2 adapter protein that promotes cyclin B degradation. Cks1 up-regulation, cyclin B down-regulation, and VSMC polyploidization were evidenced at the smooth muscle of capacitance arteries of genetically hypertensive and Goldblatt-operated rats. In addition, angiotensin II infusion dramatically increased Cks1 protein levels at capacitance arteries of normotensive rats, and angiotensin II treatment of isolated VSMC abrogated their ability to down-regulate Cks1 and maintain cyclin B protein expression in response to colcemid. Finally, transduction of VSMC from normotensive animals with a retrovirus that drives the expression of Cks1 was sufficient to alter their mitotic spindle cell cycle checkpoint status and promote an-scheduled cyclin B metabolism, cell cycle re-entry, and polyploidization. These data demonstrate that Cks1 regulates cyclin B metabolism and ploidy in VSMC and may contribute to the understanding of the phenomena of VSMC polyploidization during hypertension.

Hypertension is accompanied by changes in vascular smooth muscle cell (VSMC) growth that are specific for different vascular territories. Vascular smooth muscle hypertrophy predominates at capacitance arteries, those of high compliance, and is associated to VSMC polyploidization in hypertensive individuals (1) and animals (2–8). Tetraploid and octaploid VSMC of hypertensive rats have 2.4- and 4.8-fold, respectively, the protein content of diploid VSMC of normotensive rats (4). Additionally, on a per cell basis, polyploid VSMC express higher levels of platelet-derived growth factor A, fibronectin, and collagen than their diploid counterparts (9). Importantly, the hypertrophy of vascular smooth muscle at capacitance arteries causes arterial stiffness and promotes left ventricular overload and altered coronary blood perfusion (10).

Several stimuli, including catecholamines (11–13), angiotensin II (3, 7, 14), deoxycorticosterone/salt (11, 15, 16), and nitric oxide synthase blockade (3, 7) are known to induce VSMC polyploidization. The effects of angiotensin II on VSMC ploidy have been extensively characterized. Infusion of angiotensin II in rats promotes VSMC polyploidization at large arteries (14). Additionally, activation of the renin-angiotensin system by occlusion of a renal artery, or Goldblatt’s operation, increases aortic VSMC ploidy in these animals (17). Moreover, treatment of spontaneously hypertensive rats (SHR) and its derivative strain stroke-prone SHR with angiotensin converting enzyme inhibitors or angiotensin II AT1 receptor blockers inhibits VSMC polyploidization (4, 18, 19). However, despite extensive investigation, the molecular mechanism(s) underlying VSMC polyploidization has eluded characterization.

Mammalian cells are protected from cell cycle re-entry at mitosis by the activity of the mitotic spindle cell cycle checkpoint (20–22). This pathway delays the exit from mitosis if the chromosomal segregation cannot be properly completed by preventing the inactivation of the M-phase promoting complex (Cdc2, cyclin B, and associated proteins) (23–25). Recent data indicate that, in mammalian cells, M-phase growth arrest is accomplished in part by down-regulation of Cks1 (26), a Cdc2 adapter protein that promotes cyclin B metabolism (27–29). Cells in which the mitotic checkpoint fails to down-regulate Cks1 expression cannot maintain cyclin B protein expression and M-phase growth arrest, and are predisposed to undergo cell cycle re-entry and polyploidization (28).

The incidence of polyploidy in vascular smooth muscle of hypertensive individuals prompted us to investigate the activity of the mitotic spindle cell cycle checkpoint in VSMC. The status of this pathway was studied in cultures of VSMC isolated from multiple vascular beds of normal and hypertensive rats. We provide functional and biochemical evidence of a specific mitotic spindle cell cycle checkpoint defect in cultures of VSMC isolated from capacitance arteries of SHR. These cells express high levels of Cks1 protein and fail to down-regulate Cks1 in response to mitotic inhibitors. VSMC isolated from resistance arteries of SHR had low Cks1 expression and normal mitotic checkpoint status. Treatment of SHR with the angiotensin converting enzyme inhibitor captopril reduced Cks1 and...
ploidy levels in aortic smooth muscle. Furthermore, activation of the renin-angiotensin system in the normotensive rat strain WKY by renal artery clipping, or angiotensin II infusion, induced Cks1 protein levels and VSMC polyploidization at aortic smooth muscle. In addition, treatment of primary cultures of WKY VSMC with angiotensin II induced Cks1 up-regulation and failure to control cyclin B expression in response to a mitotic spindle inhibitor. Finally, ectopic expression of Cks1 in VSMC isolated from normotensive rats reproduced the altered mitotic spindle cell cycle checkpoint phenotype observed in VSMC of hypertensive rats. In summary, these data demonstrate that Cks1 regulates VSMC ploidy and suggest that Cks1 up-regulation may contribute to the phenomena of VSMC polyploidization during hypertension.

EXPERIMENTAL PROCEDURES

Animals—WKY and SHR were from Charles River, Wilmington, MA. Harlan Sprague-Dawley and Zucker lean rats were from Harland Harlan Sprague-Dawley, Indianapolis, IN. Two-kidney one-clip Goldblatt and sham-operated WKY rats were from Taconic, Germantown, NY. Goldblatt-operated rats (10 weeks old) were sacrificed 2 months after surgery. Captogrel (Rbl) was given in tap water ad libitum for 2 months to 8-week-old SHR rats at a dose of 100 mg/kg/day. Angiotensin II (Sigma) was infused at 50 ng/kg/min using subcutaneous osmotic pumps (Alza, Palo Alto, CA). Blood pressure was determined using tail cuffs and a programmed electrophysymomanometer (Narco, Austin, TX). For immunohistochemistry, 5-μm formalin-fixed paraaffin-embedded arterial sections were deparaffinized in xylene, rehydrated, and processed for periodic acid-Shiff-hematoxylin staining. Immunohistochemistry was carried out using Vectastain-ABC kits (Vector, Burlingame, MA) and antibodies (Santa Cruz Biotechnologies, Santa Cruz, CA) at dilution 1:50 (Cks1, MPP2) or 1:100 (cyclin B, proliferating cell nuclear antigen (PCNA)). Cells from at least five biopsies were scored for quantitative analysis by two independent observers. Pooled data were analyzed for statistical significance using ANOVA followed by Fisher’s exact test (30).

Isolation, Infection, and Culture of VSMC—VSMC and vascular fibroblasts were isolated as described previously (31). After isolation, cells were seeded at a density of 3,500 cells/cm² and grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum. VSMC of hypertensive rats. In summary, these data demonstrate that Cks1 regulates VSMC ploidy and suggest that Cks1 up-regulation may contribute to the phenomena of VSMC polyploidization during hypertension.


data were analyzed for significance using ANOVA followed by Fisher's exact test (30).


tables prepared as described previously (26). Western blots were performed using Cks1 (Santa Cruz), cyclin B1 (Santa Cruz), and β-actin (Sigma) antibodies at dilutions 1:100, 1:500, and 1:10,000, respectively. For quantification, gels were scanned using an LKB Ultrascan XL densitometer and GelScan XL software (LKB, Uppsala, Sweden). Western blots were performed using Cks1 (Santa Cruz), cyclin B1 (Santa Cruz), and β-actin (Sigma) antibodies at dilutions 1:100, 1:500, and 1:1,000, respectively. For quantification, gels were scanned using an LKB Ultrascan XL densitometer and GelScan XL software (LKB, Uppsala, Sweden). When cell cycle time-course studies were carried out, the onset of protein down-regulation was determined as the experimental time point at which a >50% decrease in protein levels was detected. Significant differences in the distribution of data were determined by G test (30). G values were compared using a χ² distribution with one degree of freedom. A p < 0.05 was considered significantly different.

RESULTS

VSMC from Prehypertensive Animals Are Predisposed to Polyploidization—The ability of VSMC to arrest growth at M phase in response to mitotic spindle depolymerizing agents was analyzed using a flow cytometry-based functional assay (22). Primary cultures were generated from VSMC isolated from multiple vascular territories of 3-week-old SHR. This age was selected because it precedes the onset of VSMC polyploidization and hypertrophy in the SHR (4). Cultures of skin and vascular fibroblasts isolated from SHR as well as VSMC isolated from age-matched WKY, Harlan Sprague-Dawley, and Zucker lean rats were also investigated. Cells were incubated in media containing colcemid for an incubation period equal to 2 PDL, in order to compare equal number of mitotic events among cell groups, and the percentage of cells with >4 N DNA content determined using flow cytometry (Table I). A small fraction of polyploid SHR skin fibroblasts was detected (5%) of >N cells). This number is within the range previously found in mouse and human fibroblasts (21, 22). Strikingly, VSMC isolated from multiple vascular beds showed significant differences in their ability to control their DNA ploidy. Cultures of VSMC from capacitance arteries of SHR, such as carotid arteries or aorta, progressed to significantly higher levels of polyploidy than skin fibroblasts (14–28% of >4 N cells, p < 0.01). In contrast, VSMC from mesenteric superior arteries,

| Table I | VSMC isolated from capacitance arteries of SHR are predisposed to polyploidization |
|------------------|---------------------------------|
| **Cell population** | **DNA content > 4N after 2 PDL in colcemid (%)** | **n** | **h** |
| SHR | Skin fibroblasts | 5 ± 0.7 | 4 52 |
| VSMC carotid arteries | 25 ± 3.1* | 3 68 |
| VSMC proximal aorta | 28 ± 3.3* | 5 73 |
| Fibroblasts proximal aorta | 8 ± 0.5 | 3 71 |
| VSMC distal thoracic aorta | 14 ± 2.5* | 3 67 |
| VSMC mesenteric artery | 6 ± 0.6 | 4 72 |
| VSMC mesenteric branches | 7 ± 1.0 | 3 75 |
| WKY | VSMC proximal aorta | 4 ± 0.9 | 5 86 |
| VSMC mesenteric artery | 7 ± 0.5 | 4 90 |
| WKY-LXSN/pBabe | 5 ± 0.9 | 3 78 |
| WKY-pBabe-p53RScmut | 24 ± 0.9* | 3 66 |
| WKY-LXSN-E6 | 8 ± 1.2 | 3 64 |
| Sprague-Dawley | VSMC proximal aorta | 6 ± 1.4 | 3 76 |
| VSMC proximal aorta | 5 ± 0.8 | 3 77 |

extracts prepared as described previously (26). Western blots were performed using Cks1 (Santa Cruz), cyclin B1 (Santa Cruz), and β-actin (Sigma) antibodies at dilutions 1:100, 1:500, and 1:1,000, respectively. For quantification, gels were scanned using an LKB Ultrason XL densitometer and GelScan XL software (LKB, Uppsala, Sweden). Western blots were performed using Cks1 (Santa Cruz), cyclin B1 (Santa Cruz), and β-actin (Sigma) antibodies at dilutions 1:100, 1:500, and 1:1,000, respectively. For quantification, gels were scanned using an LKB Ultrason XL densitometer and GelScan XL software (LKB, Uppsala, Sweden). Western blots were performed using Cks1 (Santa Cruz), cyclin B1 (Santa Cruz), and β-actin (Sigma) antibodies at dilutions 1:100, 1:500, and 1:1,000, respectively. For quantification, gels were scanned using an LKB Ultrason XL densitometer and GelScan XL software (LKB, Uppsala, Sweden). Western blots were performed using Cks1 (Santa Cruz), cyclin B1 (Santa Cruz), and β-actin (Sigma) antibodies at dilutions 1:100, 1:500, and 1:1,000, respectively. For quantification, gels were scanned using an LKB Ultrason XL densitometer and GelScan XL software (LKB, Uppsala, Sweden).
mesenteric branches, and adventitial fibroblasts from the SHR proximal aorta maintained a low ploidy content (6–7% of 4N cells). VSMC isolated from capacitance and resistance arteries of the normotensive strains WKY, Harlan Sprague-Dawley, and Zucker lean rats also demonstrated active growth arrest at 4N (5–6% of 4N cells). Incubation of SHR proximal aorta VSMC with nocodazole or higher concentrations of colcemid (up to 1 μg/ml) did not result in sustained 4N growth arrest (data not shown). Additionally, in the absence of mitotic inhibitors, only 1–2% of 4N cells were detected in all cell groups (data not shown). Importantly, the phenotypic differences observed were independent of the proliferation rate, since cells were analyzed at an equal number of PDL (22). Additionally, some cell populations, such as SHR skin fibroblasts, with a higher growth rate than VSMC, displayed normal ability to control DNA ploidy (Table I). In addition, infection of VSMC isolated

FIG. 1. Unscheduled degradation of cyclin B in VSMC from capacitance arteries of SHR. Western blot of cyclin B and β-actin proteins in extracts prepared from synchronized cultures of VSMC and skin fibroblasts isolated from 3-week-old SHR and WKY rats. a and b, confluent cell cultures (4 × 10⁴ to 5 × 10⁵ cells/cm²) were synchronized by a 2-day incubation in low serum medium (0.5% calf serum), incubated at low density (1 × 10⁴ to 2 × 10⁴ cells/cm²) in 10% FBS in the absence (no colcemid) or presence (colcemid) of 100 ng of colcemid/ml, and harvested at the indicated intervals. c–i, cells were synchronized as above and incubated with 100 ng/ml of colcemid. A representative Western blot of three to five experiments per each cell population is shown.
from the proximal aorta of WKY rats with a retroviral vector that drives the expression of a tumor suppressor gene p53RSC mutant (WKY-pBabe-p53RSCmut; RSC, relaxed mitotic spindle checkpoint allele) or the human papilloma virus E6 protein (WKY-LXSN-E6) generated cultures of VSMC with high proliferative rates. However, only those VSMC infected with the mitotic checkpoint deficient p53RSC vector accumulated an elevated fraction of polyploid cells (24%, p < 0.001 related to SHR skin fibroblasts).

The results shown in Table I suggested that VSMC from capacitance arteries of SHR undergo polyploidization in culture because of a defective mitotic spindle cell cycle checkpoint. This pathway delays the degradation of mitotic proteins, such as cyclin B, if the segregation of chromosomes cannot be properly completed (21). In exponentially growing cells, cyclin B protein oscillates during the cell cycle. The level of this protein raises at the transition between the G2 and M cell cycle phases and declines sharply when cells exit M-phase. Activation of the mitotic spindle cell cycle checkpoint by spindle depolymerizing agents inhibits cyclin B degradation and originates a prolonged, although not permanent, period of cyclin B protein expression (26). We observed that, in synchronized cultures of SHR skin fibroblasts, cyclin B protein levels oscillated in 48-h cycles (Fig. 1a), indicating normal entry and exit from M phase. When colcemid was added to the cultures, cyclin B protein was detected up to 120 h after cell cycle entry, indicating transient mitotic arrest, and thus, normal mitotic spindle checkpoint activity. In the absence of inhibitor, no differences in the expression of cyclin B protein (48-h peaks) were observed among cultures of VSMC from multiple vascular territories (Fig. 1b, no colcemid and data not shown). However, incubation of these cells with colcemid revealed striking differences in their ability to regulate cyclin B protein expression. Fig. 1 (b–i) shows a representative gel for each vascular territory investigated. In order to quantify these results, the time of onset of cyclin B down-regulation was determined as the experimental time point at which a >50% decline in cyclin B levels was detected by gel densitometry. A summary of these experiments is shown in Table II. Significant differences in the onset of cyclin B down-regulation were observed between cultures of VSMC isolated from capacitance arteries (proximal aorta VSMC, carotid arteries VSMC, and distal thoracic aorta VSMC) of SHR rats and skin fibroblasts isolated from the same animals (p < 0.05). Thus, VSMC from capacitance arteries of SHR failed to delay cyclin B degradation in response to the mitotic inhibitor, indicating defective mitotic checkpoint activity. In contrast, normal mitotic checkpoint activity was found in cultures of VSMC isolated from mesenteric arteries and branches of SHR (Fig. 1h and i and Table II). Significant differences were found also between cultures of VSMC from capacitance arteries of SHR and their WKY counterparts (p < 0.05). In addition, VSMC isolated from aortas of Harlan Sprague-Dawley rats demonstrated sustained delay of cyclin B metabolism in response to colcemid (data not shown). Taken together, the results shown in Fig. 2 and Tables I and II demonstrate a defective mitotic spindle cell cycle checkpoint in cultures of VSMC isolated from capacitance arteries of rats predisposed to hypertension.

Cks1 Is Up-regulated in the Smooth Muscle of Hypertensive Rats—It has been shown that Cks1 plays a key role in the control of the progression through M phase in mammalian cells (26). The expression of this protein was investigated using Western blot analysis in VSMC freshly isolated from the vascular smooth muscle of WKY and SHR rats. Strikingly, a gradient of Cks1 was observed along the vascular tree (Fig. 2a), with significantly higher levels of protein at capacitance arteries of SHR rats than in their WKY counterparts (266 ± 6% increase in SHR, p < 0.003; Fig. 2a and data not shown).

Additionally, immunohistochemistry experiments demonstrated that Cks1 up-regulation is specific for the medial vascular smooth muscle layer, with background Cks1 expression levels at intima and adventitia (Fig. 2b). Moreover, although Cks1 protein was expressed at higher levels in aortic VSMC of SHR rats (33% versus 11% of positively stained cells, p < 0.003), higher levels of cyclin B protein were found in age-matched WKY rats (78% versus 34% of positively stained cells, p < 0.001). In addition, the mitotic marker M-phase protein 2 (MPP2) was expressed at higher levels in SHR than in WKY rats (36% versus 12% MPP2-positive cells, p < 0.001), whereas no differences were observed in the level of PCNA protein between both rat strains (Fig. 2b). Thus, these results demonstrate that enhanced Cks1 expression accompanies cyclin B down-regulation and mitotic progression in VSMC of aortas of SHR rats. Aorta SHR VSMC have been previously shown to undergo polyploidization and hypertrophy in vivo (4, 5, 33, 34); our data support the hypothesis that mitotic checkpoints play a role in the regulation of VSMC growth in vivo.

In order to control for genetic divergence between the SHR and WKY strains, two series of experiments were conducted. First, SHR rats were treated for 2 months with captopril, an angiotensin converting enzyme inhibitor. Aortic VSMC were isolated from sham- and captropril-treated SHR rats, and Cks1 and cell ploidy levels determined. A decrease (59 ± 7%, p < 0.004) in the polyploid VSMC fraction in captropril-treated animals was accompanied by a reduction (64 ± 3%, p < 0.001) in Cks1 protein levels (Fig. 2c and data not shown). In the second group of experiments, the renin-angiotensin system was activated in WKY rats clipping the left renal artery (Goldblatt's surgery). This procedure has been shown previously to induce VSMC hypertrophy and polyploidization at large arteries (17). Enhanced polyploidization (262 ± 21%, p < 0.006) and increased expression of Cks1 protein (493 ± 18%, p < 0.002) were observed in Goldblatt WKY rats (Fig. 2d and data not shown).

Furthermore, aortic VSMC of Goldblatt-operated animals demonstrated lower cyclin B staining (62% decrease, p < 0.001) and higher MPP2 expression (649% increase, p < 0.001) than sham-operated animals (Fig. 2e). Thus, enhanced expression of Cks1 accompanied mitotic progression and polyploidization in an experimental model of renovascular hypertension, whereas in-
FIG. 2. Elevated Cks1 levels are associated with VSMC polyploidization. a, Western blot of Cks1 and β-actin in VSMC freshly isolated from SHR or WKY rat arteries (1 × 10^6 cells/lane). The figure is representative of three blots. Densitometry was carried out using an LKB densitometer. b, Immunohistochemistry of Cks1, cyclin B, MPP2, and PCNA in arterial sections (×250) of 3-week-old WKY and SHR. For quantification, cells from at least five biopsies were scored for quantitative analysis by two independent observers. Pooled data were analyzed for significance using ANOVA followed by Fisher's test (30). Other details as indicated under “Experimental Procedures.” c, Western analysis (upper panel) and ploidy content (lower panel) in VSMC freshly isolated from the proximal aorta of control SHR and captopril-treated SHR. Ploidy content was determined by flow cytometry of three preparations (8–12 rats) of propidium iodide-stained freshly isolated cells. Bars show percentage of cells.
terference with the renin-angiotensin system reduced Cks1 expression and polyploidization in hypertensive animals.

These experiments suggested that angiotensin II may induce VSMC polyploidization by up-regulating Cks1. This hypothesis was investigated by the infusion of sub-pressor concentrations of angiotensin II in WKY rats using subcutaneous osmotic pumps. Short term angiotensin II-treatment caused dramatic up-regulation of Cks1 at capacitance arteries (Fig. 4a). Immunohistochemistry revealed a 770% increase in Cks1-positive cells at the aorta smooth muscle related to untreated controls (p < 0.0001). Furthermore, in the absence of angiotensin II, colcemid induced lower and significantly delayed expression of Cks1 in cultures of WKY VSMC (Fig. 3c, p < 0.005). However, when angiotensin II was added to the media, peaks of Cks1 and cyclin B protein expression in these cells were observed at 48 h after cell cycle entry, despite the presence of the mitotic inhibitor (Fig. 3, b and c). Thus, these results demonstrate that angiotensin II induces VSMC Cks1 up-regulation and failure to arrest the progression of M phase in response to a mitotic inhibitor.

**Ectopic Expression of Cks1 Is Sufficient to Promote VSMC Polyploidization**—To test a direct effect of Cks1 on VSMC ploidy content, human Cks1 was expressed in VSMC isolated from the aorta of normotensive rats using a retroviral vector (pBabe-VSMC). Cultures of pBabe and pBabe-Cks1 (pBabe-Cks1) (26). Control VSMC were infected with an empty vector (pBabe-VSMC). Cultures of pBabe and pBabe-Cks1 VSMC were then growth arrested at G0, allowed to enter the cell cycle synchronously in the presence or absence of colcemid, and the levels of Cks1 and cyclin B proteins determined at multiple time points. Cks1 expression in colcemid-treated VSMC cultures was lower and significantly delayed in pBabe-VSMC (p < 0.05), but not in pBabe-Cks1-VSMC, relative to untreated VSMC (Fig. 4a). Additionally, a significant delay in the onset of cyclin B degradation was observed in colcemid treated pBabe-VSMC, but not in pBabe-Cks1-VSMC (Fig. 4a, p < 0.05). Thus, cultures of Cks1-transduced VSMC demonstrated a defective mitotic spindle cell cycle checkpoint status, with its corresponding biochemical alterations in the regulation of Cks1 and cyclin B proteins (Fig. 4a), that resembles angiotensin II-treated WKY VSMC (Fig. 3, b and c) or SHR VSMC (Figs. 1 (b–d) and 3c). Furthermore, flow cytometry of total DNA content (PI staining) versus newly synthesized DNA (BrdUrd incorporation) demonstrated that pBabe-Cks1-VSMC are able to undergo cell cycle re-entry at 4 N DNA content either in the presence of colcemid (Fig. 4b) or following cell confluence (Fig. 4c). Finally, the polyploidization rate of cultures of pBabe-VSMC and pBabe-Cks1-VSMC were investigated by karyotypic analysis. pBabe-Cks1-VSMC, but not pBabe-VSMC, accumulated a significantly higher fraction of tetraploid (4 N) and octoploid (8 N) cells than their parental WKY VSMC cultures when incubated for 2 PDLs in the presence of colcemid (Fig. 4d, p < 0.0001). Thus, these data demonstrate that unregulated Cks1 expression is sufficient to promote VSMC polyploidization. In addition, pBabe-Cks1 VSMC demonstrated a higher rate of spontaneous polyploidization in culture than pBabe-VSMC (Fig. 4e), although the difference was only marginally significant (p < 0.06).

In summary, we have investigated a molecular mechanism of control of ploidy in VSMC of hypertensive animals. Several findings are reported here. First, VSMC isolated from prehypertensive animals are predisposed to undergo polyploidization due to an altered mitotic spindle cell cycle checkpoint status. Second, angiotensin II up-regulates Cks1. Third, ectopic expression of Cks1 is sufficient to abrogate the mitotic spindle cell cycle checkpoint in VSMC. Collectively, these studies provide the first evidence for a molecular mechanism of VSMC polyploidization in hypertensive animals.
Fig. 4. Ectopic expression of Cks1 causes VSMC polyploidization. a, Western blot of cyclin B, β-actin, and Cks1 in WKY aortic VSMC stably infected with vectors pBabe (empty control) and pBabe-Cks1. Data are representative of four experiments. Other experimental details as in Fig. 1. b, flow cytometry of DNA content in pBabe- and pBabe-Cks1 VSMC. Asynchronous cell cultures at passages 1–3 were incubated in the absence or presence of 100 ng/ml colcemid for 1 PDL. PDL for pBabe VSMC and pBabe-Cks1 VSMC were 68 and 52 h, respectively. Data are representative of three independent experiments. c, Flow cytometry of DNA content in confluent pBabe- and pBabe-Cks1 VSMC. Data are
polyploidization. These results extend previous observations by others (2, 6, 8) that reported a higher level of polyploidization in culture of SHR VSMC than WKY VSMC. In contrast, a previous report indicated that the rate of polyploidization in culture was higher in WKY VSMC than in SHR VSMC (32). However, when these authors tested VSMC from another two normotensive rat strains, Harlan Sprague-Dawley and Fisher rats, lower polyploidization rates than in SHR VSMC were found, which suggests a phenotypic divergence of the WKY colony investigated.

Our data support also previous observations indicating a role for Cks1 in the control of M phase in mammalian cells. It has been shown that Cks1 is a target of the mitotic spindle cell cycle checkpoint pathway, and that down-regulation of Cks1 is essential to promote mitotic arrest in mammalian cells (26). However, the mechanism of action of Cks1 at mitosis is not completely understood. It is known that Cks1 and related proteins work as adapters that modulate substrate recognition by Cdc2 (35–37). Importantly, Cks1 may drive the activation by Cdc2 of the cyclin B degradation machinery at mitosis. Cks1-depleted Xenopus oocytes cannot undergo Cdc2-dependent phosphorylation of the Cdc27 component of the cyclosome, the ubiquitin ligase activity that targets cyclin B for degradation (36). Furthermore, a recent study showed that Cks1 plays a yet undefined role in the activation of the proteasome, the proteolytic complex that degrades cyclosome-ubiquitinated cyclin B (37). Thus, we hypothesize that up-regulation of Cks1 in hypertensive VSMC uncouples the mitotic spindle cell cycle checkpoint with the cell cycle regulatory machinery at M phase, and, consequently, abrogates the ability of these cells to control cyclin B metabolism and arrest the progression of mitosis (Fig. 5). Further studies on the regulation of Cks1 expression and activity by angiotensin and other growth factors may contribute to a better understanding of the pathways that modulate the VSMC phenotype during hypertension (38). Importantly, the fact that growth factors may control the expression of proteins required for M-phase progression in VSMC was postulated previously (39), but direct evidence has been lacking until now.

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FIG. 5. Model of mitotic spindle cell cycle checkpoint activity and Cks1 role in VSMC polyploidization. Cks1 is required for the degradation of cyclin B protein at the exit from mitosis. Mitotic spindle cell cycle checkpoint signals originate at kinetochore/microtubule interaction points activate a pathway that down-regulates Cks1 expression, delays cyclin B degradation, and arrests the progression of M phase. In VSMC with altered mitotic spindle checkpoint status, Cks1 levels are not down-regulated; mitotic exit may proceed in the absence of segregation and karyokinesis, leading to cell cycle re-entry and polyploidization.
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