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EXPRESSION OF SOLUBLE AND INSOLUBLE FIBRONECTIN IN RAT AORTA: EFFECTS OF ANGIOTENSIN II AND ENDOTHELIN-1

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SUMMARY: This study has investigated the influence of the vasoconstrictor peptides angiotensin II (Ang II) and endothelin-1 (ET-1) on fibronectin expression by vascular smooth muscle cells (VSMC). In confluent, quiescent cultures of VSMC, Ang II and ET-1 elevated fibronectin mRNA levels in a time- and dose-dependent fashion. ET-1 and Ang II also induced a time-dependent expression of immunoreactive fibronectin in cultures of aortic organoids, and for both peptides the fibronectin immunoreactivity was most prominent within those medial smooth muscle cell layers close to the vessel lumen. Immunoprecipitation of biosynthetically labelled fibronectin elaborated by cultured VSMC revealed a predominant expression of soluble fibronectin in response to Ang II, whereas for ET-1 the newly synthesized fibronectin was predominantly incorporated into the extracellular matrix deposit of the cells. These findings indicate that Ang II and ET-1 may exert disparate effects on smooth muscle cell phenotype and migration.

Fibronectin is a dimeric glycoprotein found in the extracellular matrix of most tissues. It is thought to play a role in various biological events such as adhesion and migration of cells, wound repair and oncogenesis (1,2). The glycoprotein is produced from transcripts of a single gene which is subject to alternative splicing, leading to different isoforms of fibronectin (3). Fibronectin can bind to other components of the extracellular matrix, as well as to specific cellular receptors (4). These interactions influence the structural and mechanical properties of the matrix and the phenotype of adherent cells. In cultured vascular smooth muscle cells, fibronectin has been shown to specifically induce phenotypic changes (i.e. conversion from the contractile to the secretory, proliferative phenotype) (5) that resemble those occurring within the

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Abbreviations: VSMC, vascular smooth muscle cells; Ang II, angiotensin II; ET-1, endothelin-1; MEM, minimal essential medium; FCS, fetal calf serum; BSA, bovine serum albumin; PDS, plasma-derived serum; PBS, phosphate buffered saline.

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vasculature in vivo in association with vascular pathologies such as hypertension and atherosclerosis (6). Therefore it was suggested that fibronectin may have a causative role in vascular hypertrophy and neointima formation (5). The vasoconstrictor peptides angiotensin II (Ang II) and endothelin (ET-1) have been demonstrated to promote vascular smooth muscle cell growth in vitro (7,8), but the effects of these peptides on fibronectin expression and synthesis have not been investigated. Therefore we have examined the effects of Ang II and ET-1 on the expression of fibronectin in both cultured rat aortic vascular smooth muscle cells (VSMC) and rat aortic organoids. The results obtained may be relevant to differential long-term biological functions of vasoconstrictor peptides in the regulation of vascular architecture in vivo.

MATERIALS AND METHODS

Culture of aortic smooth muscle cells and aortic organoids: Male, 20-week old spontaneously hypertensive rats (Charles River Wiga, Sulzfeld, Germany) were used throughout this study. The procedures for isolation, characterization and propagation of aortic VSMC have previously been detailed (9). Cultures (between passages 3-5) were maintained in minimal essential medium (MEM) containing 10% fetal calf serum (FCS), and prior to stimulation of VSMC with the peptide agonists, cells were rendered quiescent by serum deprivation for 48 hrs (FCS replaced by 0.1% bovine serum albumin (BSA)). For the preparation of aortic organoids, ≈ 5mm-wide rings were sliced from comparable parts of the thoracic aortae and placed into 3 ml of MEM/1% plasma derived serum (PDS) in Costar 12-well multidishes. Organoids were cultured for up to seven days in the absence or continuous presence of Ang II or ET-1, with medium changes every two days. Subsequently organoids were prepared for immunohistochemistry. The functional viability of organoid cultures was tested in an organ chamber, and the contractile response to 100 mM KCl was comparable between freshly isolated and seven-day cultured organoids.

Northern blot analysis: Procedures for preparation of total RNA, electrophoresis (20 μg/lane), sequential hybridization and autoradiography have been described previously (9). Probes used in this study were partial cDNAs specific for rat fibronectin (10) and GAPDH (ATCC No 57090, Rockville,MD,USA), the latter serving as an internal control for variability in amounts of RNA loaded. Autoradiograms were analysed using an integrated video-camera and the SCREEN MACHINE programm from Macintosh. Densitometric readings of 7.9 Kb signals in each lane were normalized with respect to the corresponding densitometric readings for GAPDH- specific hybridizations.

Biosynthetic labelling and immunoprecipitation: Quiescent VSMC ($3x10^6$ cells) were preincubated in 3 ml of methionine-free culture medium containing 1% BSA for one hour before the addition of 100 μCi of [^{35}S]- methionine (Amersham, UK, 1100 Ci/mmole) and either vehicle, Ang II or ET-1 (each at 10^{-7}M). Incubation was continued for 16 hours and then supernatants were harvested and extracellular matrix deposits were isolated and solubilized according to previously described methods (11) All subsequent immunoprecipitation procedures were performed on ice. Prior to immunoprecipitation, aliquots (corresponding to 10^6 cells) of matrix lysates and supernatants were pre-cleared by incubation for 1 hour using mouse and rabbit normal serum (10 μl each) and 500 μl of Omnisorb cells (Calbiochem, LaJolla, CA, USA). Subsequently the immunoadsorbents were removed by centrifugation and supernatants were transferred to fresh Eppendorf tubes. Following addition of mouse monoclonal

antibodies (10 μg/tube) directed against human fibronectin but which cross-reacted with rat fibronectin (F 6140, Sigma, St. Louis, MO, USA), incubation was continued for 1 hour before the addition of 10 μg of second antibody (rabbit anti- mouse immunoglobulins, Dakopatts, Glostrup, DK) and 100 μl of Omnisorb. After further incubation (30 mins) the immunoadsorbents were pelleted by centrifugation, the supernatants removed and pellets washed sequentially according to methods described (12). Immunoprecipitates were electrophoresed on denaturing polyacrylamide gradient (5-10%) gels, and then gels were treated with Amplify (Amersham, UK), dried and exposed overnight to Kodak XAR 5 film at -70°C.

Immunohistochemistry: Aortic organoids were embedded in Tissue-Tek OCT (Miles Laboratories Inc.,Naperville, IL) and frozen rapidly in an isopentane bath in liquid nitrogen. Sections (≈ 5 μm) were cut in a freezing microtome, applied to polylysine-coated slides, dried and stored at -70°C. Sections were stained by the peroxidase-antiperoxidase method. To block endogenous peroxidase- activity, sections were pre-incubated in methanol/1% (v/v) hydrogen peroxide. Non-specific protein binding was eliminated by incubation of the sections for 10 mins. in normal rabbit serum (1:30 in phosphate buffered saline (PBS)) before incubation for 2 hours with specific first antibody (1:500 in PBS containing 0.1% (w/v) BSA). Sequential incubations with the second antibody (rabbit anti- mouse immunoglobulins; Dakopatts, Glostrup, DK; 1:70) and peroxidase- antiperoxidase complexes (Dakopatts; 1:70) were performed for 30 mins each. Between antibody incubation steps, sections were washed with PBS containing 0.1% BSA. Diaminobenzidine (tablets from Dakopatts) was used as the substrate for peroxidase. Sections were counterstained with haematoxyline, differentiated in 1% HCl/ethanol and dehydrated before mounting in Eukitt's mounting medium.

RESULTS

Confluent, quiescent rat aortic VSMC constitutively expressed a low level of mRNA for fibronectin (Fig. 1A). Both Ang II and ET-1 (each at 10^{-7}M) induced a rapid increase in the expression of fibronectin transcripts which reached maximal levels ≈ 7-9 hours poststimulation (Fig. 1A). Levels of fibronectin mRNA remained maximally elevated during at least a further 24 hour period of exposure to Ang II or ET-1 (data not shown). The size of the fibronectin mRNA induced by Ang II or ET-1 was ≈ 7.9 Kb in size. Peptide-induced elevation of fibronectin mRNA levels in VSMC was blocked by actinomycin D, but not by cycloheximide (data not shown). Induction of fibronectin transcripts by the vasoconstrictor peptides was dose-dependent over the tested range of concentrations (10^{-11} to 10^{-7}M), and the threshold concentration was ≈ 10^{-10}M for both Ang II and ET-1 (Fig. 1B).

Expression of immunoreactive fibronectin was minimal in organoids both immediately ex vivo (Fig. 2A) and after culture under control conditions for 3 days (Fig. 2B) and 7 days (not shown). Organoids exposed for 3 days to either Ang II or ET-1 (each at 10^{-7} M) exhibited an increased (vs. controls) expression of immunoreactive fibronectin within the medial smooth muscle cell layers (Fig. 2C and

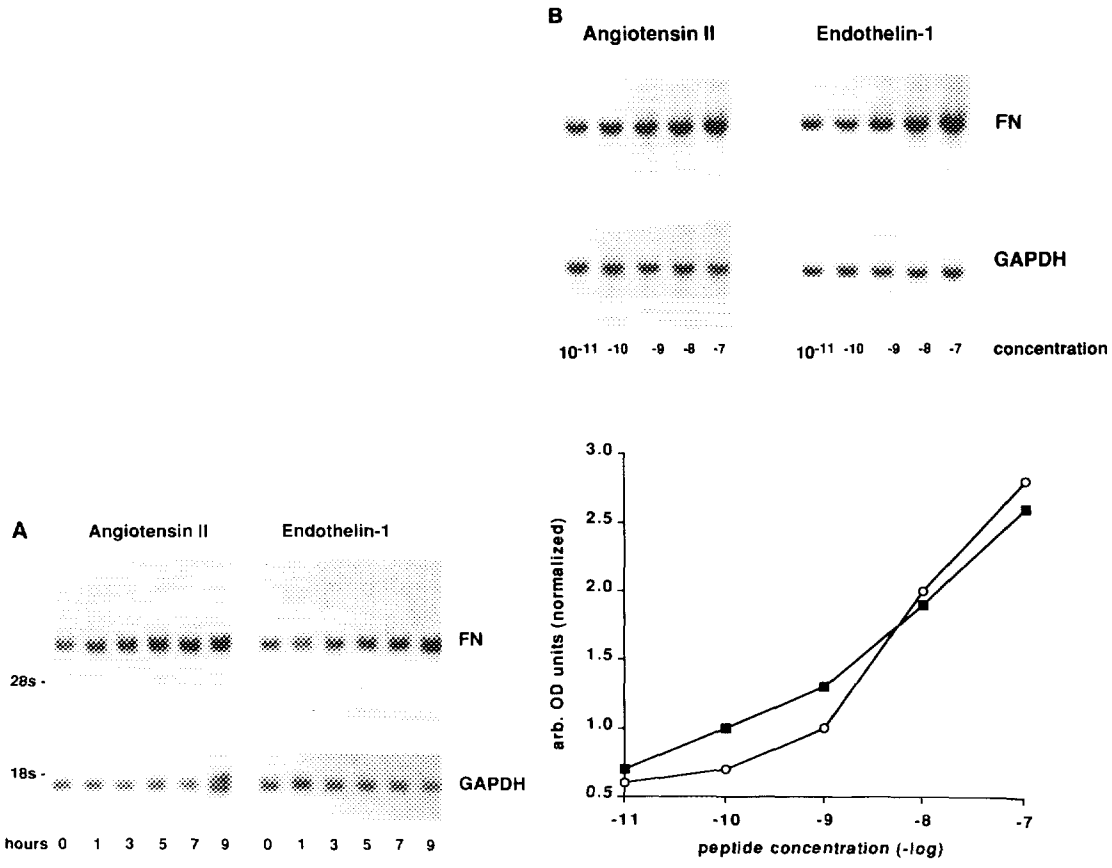

Figure 1. Panel A:- Time-dependent stimulation of fibronectin transcript (7.9 Kb) expression in VSMC by either Ang II or ET-1 (each at 10^{-7}M). A representative autoradiogram is presented, and comparable time-dependencies were observed in three separately performed experiments, each using a different VSMC isolate. **Panel B:-** Dose-dependent stimulation of fibronectin-specific mRNA (7.9 Kb) by Ang II and ET-1 (after 6 hours) in VSMC. A representative autoradiogram is shown, and the normalized values represent the mean (SD 10-15%) of data obtained in three experiments.

D). Fibronectin immunoreactivity within the media was most pronounced toward the luminal side of the vessel and weakened with progression toward the adventitia. After 7 days of exposure to Ang II or ET-1, fibronectin immunoreactivity decayed toward those levels observed in non-stimulated organoids (Fig. 2E and F). After 7 days of culture fibronectin immunoreactivity was also observed in the endothelial cell layer, and this may represent newly synthesized fibronectin and/or fibronectin adsorbed from medium-contained PDS.

To investigate whether peptide-induction of fibronectin transcripts in VSMC was accompanied by expression of glycopeptide, VSMC were biosynthetically labelled

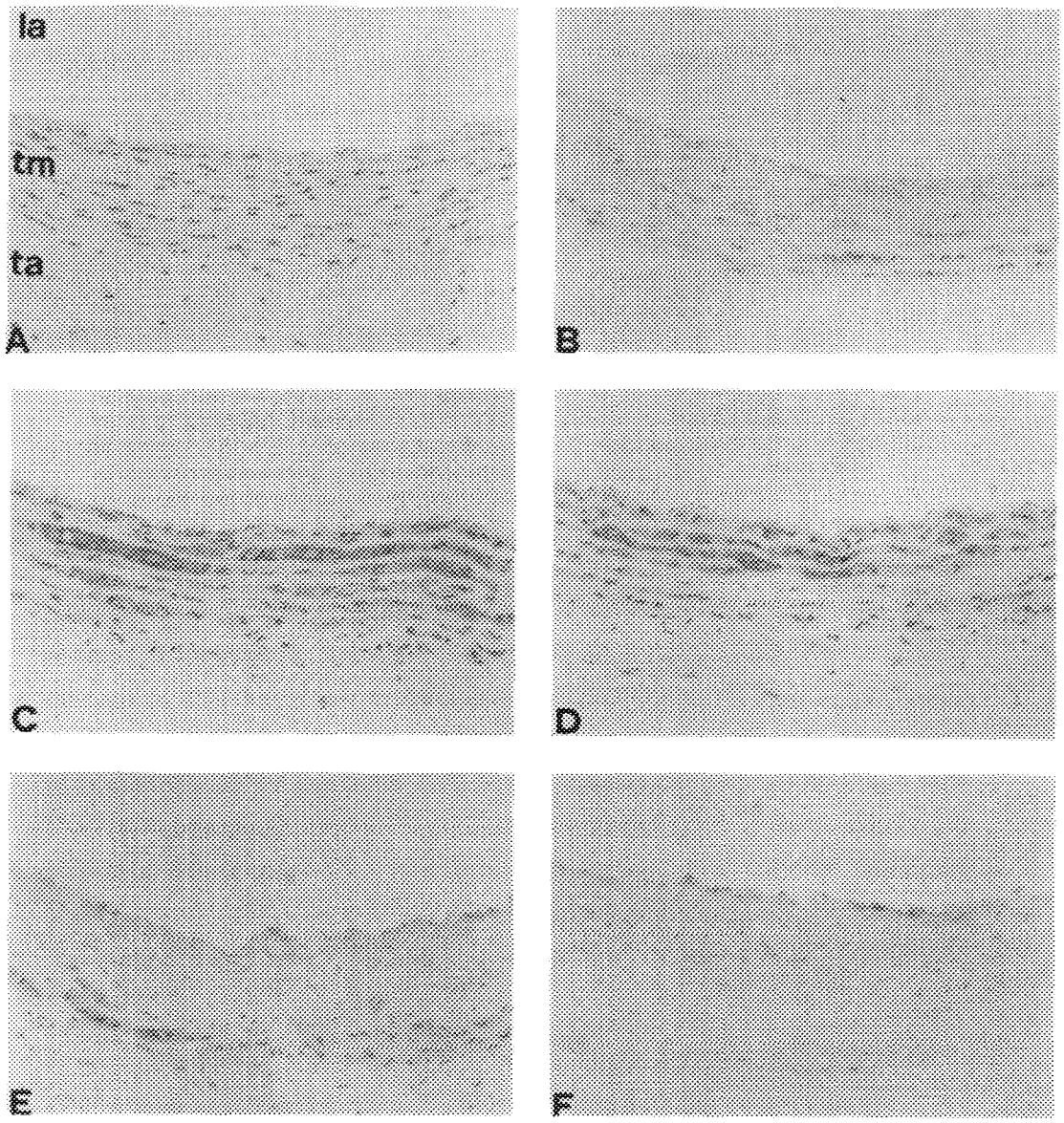

Figure 2. Induction of immunoreactive fibronectin in rat aortic organoids. Organoids were analysed immediately ex vivo (Panel A) or were incubated without (control) or with inclusion of Ang II or ET-1 (each at 10^{-7}M) in the culture medium (MEM/1% PDS) for either 3 days (Panels B, C and D) or 7 days (Panels E and F). All experimental details are described in "Methods". Magnification is X33.2. la= lumen aorta; tm= tunica media; ta= tunica adventitia. The results presented are representative of three separately performed experiments.

with [^{35}S]-methionine during culture. In spite of the low constitutive expression of fibronectin mRNA in quiescent VSMC (Fig. 1), neither supernatants (Fig. 3A) nor matrix deposits (Fig. 3B) from non-stimulated VSMC contained detectable levels of

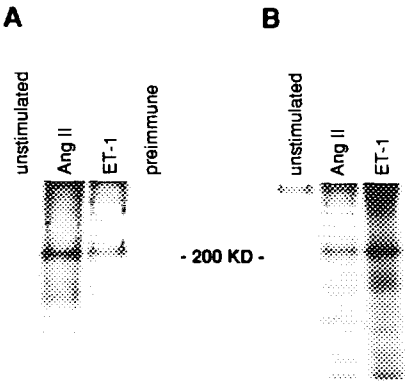

Figure 3. Immunoprecipitation of biosynthetically labelled fibronectin from medium supernates (Panel A) and matrix deposits (Panel B) from VSMC following 16 hours incubation in the absence (control) or presence of either Ang II or ET-1 (each at 10^{-7}M). Biosynthetic labelling and immunoprecipitation procedures were performed as described in "Methods". The autoradiograms are representative of two separately performed experiments.

biosynthetically labelled fibronectin glycoprotein. For VSMC exposed for 16 hours to either Ang II or ET-1, newly synthesized fibronectin was found both in medium supernatants (Fig. 3A) and in extracellular matrix deposits (Fig. 3B). However, there were distinct differences between Ang II- and ET-1-stimulated VSMC with respect to the relative abundancies of soluble and insoluble fibronectin. Compared with Ang II-stimulated VSMC, those cultures stimulated with ET-1 contained a greater abundance of fibronectin within the extracellular matrix deposit (Fig. 3B), but a lower abundance of soluble fibronectin (Fig. 3A).

DISCUSSION

A major pathological and biochemical finding in hypertensive arterial structures is an increase in arterial extracellular matrix material (13), and this also occurs as an adaptive response to arterial injury and balloon angioplasty (14). Arterial pathologies in hypertension include the migration and proliferation of vascular smooth muscle cells, and the eventual formation of neointimal layers that cause restriction of the vessel lumen (14). A variety of growth factors and vasoconstrictor peptides have been reported to stimulate the growth of smooth muscle cells and their deposition of extracellular matrix (7,15,16). Gradients of growth factors across the vessel wall are a recognized prerequisite for directed VSMC migration (17). However, the actual process of cell migration greatly depends on the presence and/or absence of

extracellular matrix components which interact with cellular surface receptors and thus enable a directed migration of cells. One such extracellular matrix component involved in the processes of cell migration and adhesion is the glycoprotein fibronectin (18).

Steady-state levels of fibronectin mRNA expression have been shown to be variable in aortae of animals with differing blood pressure (19) and with different circulating Ang II concentrations (16). Such data identify pressure-load and the renin/angiotensin-system as important intrinsic regulators of the vessel wall structure. We observed that stimulation of cultured VSMC with the vasoconstrictor peptides Ang II and ET-1 results in elevated levels of fibronectin transcript expression as well as increased de novo synthesis of the glycoprotein. Our findings with respect to the effects of Ang II in cultured VSMC are consistent with a previous demonstration of in vivo induction of fibronectin mRNA expression in the aortae of rats perfused with Ang II (19). Both Ang II- and ET-1- mediated induction of the 7.9 Kb mRNA encoding fibronectin were insensitive to inhibition by cycloheximide, thus suggesting direct transcriptional activation of the gene by the vasoconstrictor peptides. However, since we also observed that the constitutive expression of fibronectin mRNA in quiescent VSMC was not accompanied by de novo synthesis and secretion of the glycoprotein, a post-transcriptional and/or cell cycle-dependent control of fibronection expression is indicated. Cell-cycle control of fibronectin synthesis has been demonstrated in cultured fibroblasts (20).

In order to attach some physiological relevance to the in vitro (in cultured VSMC) effects of Ang II and ET-1 on fibronectin transcript expression and glycoprotein synthesis, we studied the effect of these vasoactive peptides in cultures of aortic organoids. In this ex vivo model, smooth muscle cells are maintained in their proper tissue microenvironment but are not subject to in vivo systemic influences. The responses of organoid cultures to externally applied agonists are thus likely to resemble, although cannot exactly mimic, those responses obtained in vivo. We found that Ang II and ET-1 increased, albeit transiently, the expression of immunoreactive fibronectin within medial smooth muscle cell layers. The observed time-dependent decay (c.f. 3 day- vs. 7 day-stimulated organoids) in expression of immunoreactive fibronectin cannot be attributed to an aging effect, since organoids cultured under control conditions for 4 days and then exposed to Ang II or ET-1 for 3 days also exhibited increased (vs. 7 day controls) fibronectin immunoreactivity (data not shown).

In the experimental model of arterial angioplasty, namely balloon-catheterization, it has been demonstrated that medial smooth muscle cells start to migrate from the media toward the intima $\approx$ 4 days after ballooning (6). Our

observation of increased fibronectin expression in aortic organoids within 3 days of stimulation suggests that this glycoprotein may play some role in the initial phase of induction of smooth muscle cell migration in vivo. Using Western blotting techniques to examine levels of fibronectin in protein extracts from rat aortic rings, a clear link between hypertension and elevated fibronectin expression was established (21). However, neither the distribution of the glycoprotein within the vessel wall nor candidate stimuli for fibronectin expression were investigated. The present study has demonstrated that following stimulation of aortic organoids with Ang II or ET-1, immunoreactive fibronectin was predominantly located within those medial smooth muscle cell layers close to the vessel lumen. Fibronectin immunoreactivity within the endothelial cell layer and in smooth muscle cell layers close to the adventitia was relatively weak. The concentration gradient of fibronectin expression within peptide-stimulated vessel organoids may be indicative of a role for fibronectin in directed migration of smooth muscle cells (22).

An important finding is that in cultured VSMC, Ang II preferentially stimulated synthesis of soluble fibronectin whereas the fibronectin synthesized in response to ET-1 was preferentially incorporated into the extracellular matrix. For rat cardiac myocytes and aortic VSMC the 7.9 Kb mRNA for fibronectin has been demonstrated to represent a heterogeneous population of different mRNA splicing isoforms which possibly encode glycoproteins with differing compositions of functional domains (23,24). We cannot at present determine whether a heterogenous composition of the 7.9 Kb mRNA induced by Ang II and ET-1 accounts for the differential effects of these vasoconstrictors on the synthesis of soluble and insoluble fibronectin. Furthermore, Ang II and ET-1 may induce different patterns of expression of matrix molecules and/or cell surface receptors to which fibronectin can bind (1,2). Insoluble fibronectin (i.e. matrix deposited) promotes the formation of focal contact points and anchorage of cells to the extracellular matrix, thereby preventing cell migration (25). In contrast, soluble fibronectin preferentially associates with extracellular matrix fibrils and in so doing promotes the migration of cells (25). Differential effects of Ang II and ET-1 on the synthesis of soluble and insoluble fibronectin may indicate distinct biological consequences of Ang II- and ET-1- mediated stimulation of fibronectin expression by smooth muscle cells.

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