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Effects of Monensin on Tropoelastin Metabolism in Vascular Smooth Muscle Cells: Monensin Causes Intracellular Degradation of Accumulated Tropoelastin

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Treatment with 80 nM monensin for 48 h resulted in impairment of tropoelastin secretion during the initial 6 h and subsequent reduction of tropoelastin synthesis from 18 to 48 h to one-tenth. The steady state level of tropoelastin mRNA started to decrease at 6 h and reached one-fifth of the control level by 48 h. A pulse-chase experiment after 24 h monensin treatment demonstrated that half of the accumulated tropoelastin in the cells was rapidly degraded within 120 min. These results indicate that the marked reduction in tropoelastin synthesis from 6 to 48 h treatment may be caused by both a reduction in the tropoelastin mRNA level and accelerated intracellular degradation of tropoelastin. Thus, monensin modulates tropoelastin expression through pretranslational and posttranslational mechanisms.

Key words: elastin, intracellular degradation, monensin, secretion, smooth muscle cell.

Monensin, a Na^+ ionophore capable of collapsing Na^+ and H^+ gradients, has gained widespread acceptance as a biochemical and biological investigative tool for studying the Golgi apparatus function, and for localizing and identifying the molecular pathways of subcellular vesicular traffic. Monensin has been shown to destroy the normal structure of the Golgi complex and to affect the posttranslational modifications of proteins (*1*).

Monensin inhibits the transport of various secretory proteins, including collagen (*2, 3*), elastin (*4*), proteoglycans (*5, 6*), and fibronectin (*2, 3, 7*). In the case of elastin, monensin has been demonstrated to repress tropoelastin secretion, which caused a reduction in its mRNA level (*4*). In this study we found that reduction of tropoelastin synthesis on monensin treatment was accompanied by elevated intracellular degradation of tropoelastin as well as the reduction in the tropoelastin mRNA level.

Chick aortic smooth muscle cells were isolated from 20-day chick embryos as described by Wollinsky and Daly (*8*) and Oakes *et al.* (*9*). The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FBS) until they reached confluency.

Primary cultures at confluency were treated with 80 nM monensin (Sigma) for 48 h in DMEM supplemented with 0.5% dialyzed FBS. The cells were labeled with 1 ml of fresh valine-free DMEM (Gibco) containing 25 μCi/ml [2,3-^{3}H]valine (1.5 TBq/mmol, Amersham) during the final 3 h of treatment. The proteins in the medium were precipitated with ammonium sulfate (176 mg/ml) in the presence of a protease inhibitor cocktail comprising 1 mM each of EDTA, NEM, and PMSF. Proteins were extracted from the cell layer with 1% SDS/10 mM EDTA, followed by heating at 100°C for 10 min. Proteins in the medium and cells were individually analyzed by 4-15% gradient SDS-PAGE in the presence of 1 mM DTT. An aliquot of each sample was taken prior to electrophoresis and its radioactivity was counted with a liquid scintillation spectrometer (Beckman LS 9800) to determine the total counts applied to the gel. After electrophoresis, the gels were stained and processed for autoradiography. For quantitative assaying, X-ray films were scanned with a densitometer (Cliniscan; Helena Laboratories). Radioactivity incorporated into tropoelastin in the medium and cells was calculated from the relative density of its band as to the combined density of all bands. Total (medium + cell) tropoelastin radioactivity was taken as the sum of the radioactivity in the medium and cells. For pulse-chase experiments, cultures were treated with 80 nM monensin for 24 h and labeled with [^{3}H]valine for the last 1 h of treatment, and then chased for 30, 60, 90, and 120 min in the presence of 1 mM cold valine. On termination of the culture, the proteins in the medium or cell layer were extracted and processed for analysis by SDS-PAGE as described above.

Total RNA was isolated from cells (*10*) and stored at −80°C. The RNA was denatured and size-fractionated by electrophoresis on a 1% agarose gel, and then blotted onto a nylon filter. Hybridization was performed as previously described (*11*). The following cDNA probes radioactively labeled by random priming (Amersham) to a specific activity of $\sim 10^8$ dpm/μg DNA were used; elastin (pTE2) (*12*) and β actin (pA1) (*13*). The filters were washed and exposed to X-ray films. The autoradiograms were scanned with a densitometer.

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Abbreviations: DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; NEM, *N*-ethylmaleimide; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecylsulfate; SSC, 0.15 M sodium chloride and 0.015 M sodium citrate.

In preliminary experiments, the major band corresponding to a molecular weight of 65 kDa indicated by arrows in Fig. 1a was identified as tropoelastin on Western blotting (*11, 14*).

Exposure of the cells to 80 nM monensin for 48 h resulted in a marked reduction of tropoelastin synthesis to one-tenth of the control level in the culture medium and cell layer fractions during 18-48 h treatment. However, during short term treatment, *i.e.* the initial 0-6 h, monensin did not affect the total tropoelastin synthesis in either the culture medium or cell layer, accompanying accumulation of tropoelastin in the cell layer (Fig. 1a). Northern blot assays revealed that monensin decreased the tropoelastin mRNA level, depending on the exposure time, to one-fifth of the control level on 24 and 48 h treatments (Fig. 1b).

In order to further elucidate the mode of action of monensin, and to determine on the site of inhibition, a pulse-chase experiment after long term (24 h) exposure to monensin was performed. The total (medium+cell) tropoelastin level at 0 time of the chase period in monensin-treated cells was lower than that in control cells (Fig. 2c). This may be due to reduced synthesis of tropoelastin and its mRNA level after 24 h treatment with monensin (Fig. 1). In control cells, the tropoelastin level increased in the medium (Fig. 2a), concomitant with the decrease in the cells during the chase period of 0-120 min (Fig. 2b), resulting in an unaltered level of total (medium+cell) tropoelastin (Fig. 2c). The results indicate that the decrease in tropoelastin in the cells was due to active secretion of tropoelastin. In contrast, in monensin-treated cells, the amount of tropoelastin in the cells had decreased by one-half at 120-min chase time (Fig. 2b), and tropoelastin in the

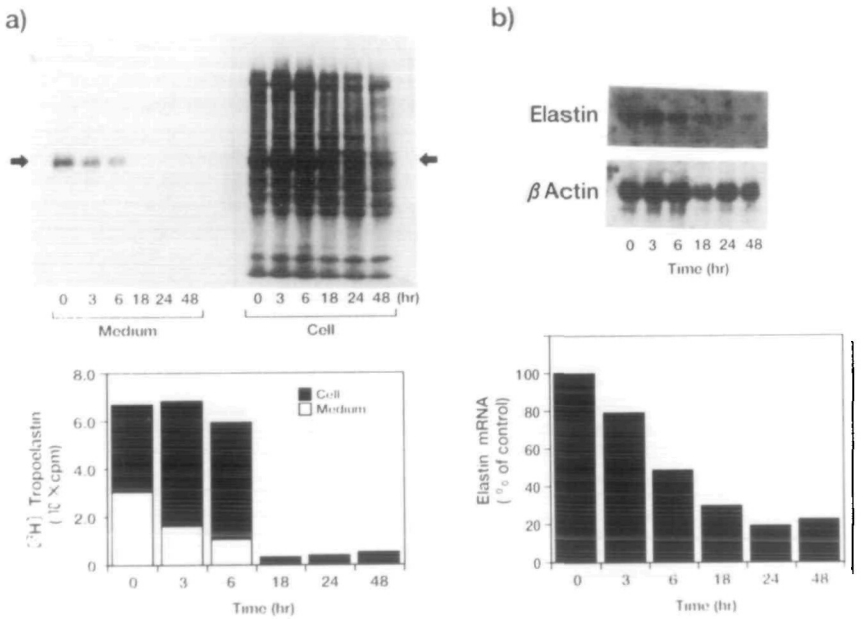

Fig. 1. **Effects of monensin on tropoelastin synthesis and the mRNA level.** Cultures were treated with 80 nM monensin for 0, 3, 6, 18, 24, and 48 h, and then labeled with [^{3}H]valine for the last 3 h of treatment. The proteins in the culture medium and cell layer were extracted, and then analyzed by SDS-PAGE, followed by autoradiography. Arrows indicate the migration position of tropoelastin. The fluorograms were scanned with a densitometer and radioactivity incorporated into tropoelastin was calculated (a). Total RNA was extracted from cells, and subjected to Northern blot analysis using elastin and β actin cDNA probes. The autoradiograms were scanned with a densitometer. The amount of elastin mRNA relative to β actin mRNA was calculated, the values given being percentages of the control (without monensin) values (b).

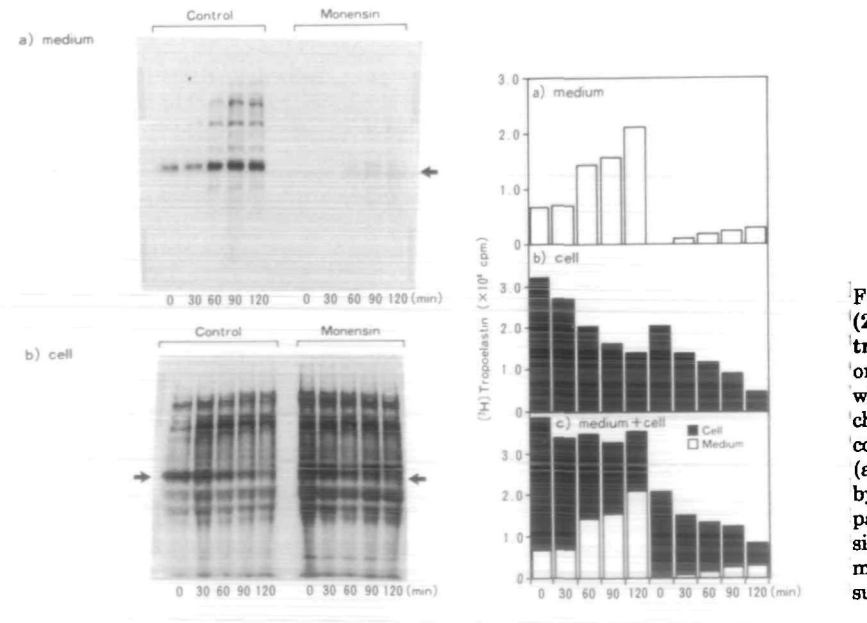

Fig. 2. **Effects of long-term monensin treatment (24 h) on the degradation of newly-synthesized tropoelastin.** Cultures were treated without (Control) or with 80 nM monensin (Monensin) for 24 h, labeled with [^{3}H]valine for the last 1 h of treatment, and then chased for 0, 30, 60, 90, and 120 min in the presence of cold valine (1 mM). The proteins in the culture medium (a) and cell layer (b) were extracted, and then analyzed by SDS-PAGE, followed by autoradiography (left panel). The autoradiograms were scanned with a densitometer, and the radioactivity of tropoelastin in the medium (a), cell (b), and medium+cell (c) was measured (right panel).

medium remained at a low level during the chase time (Fig. 2a), resulting in a reduction in the total (medium + cell) tropoelastin level of one-half (Fig. 2c). The results indicate that intracellular degradation of tropoelastin in the monensin-treated cells may have occurred during the chase period of 120 min.

The present study demonstrated that monensin impairs the secretion of tropoelastin, and reduces tropoelastin synthesis and its mRNA, which confirmed the previously reported results (*4*). Our results also demonstrated that monensin induced the degradation of intracellular tropoelastin, which may participate, at least in part, in the decrease in tropoelastin synthesis caused by monensin. The mechanism by which monensin stimulates the intracellular degradation of tropoelastin is not clear at present. Since elective intracellular receptors involved in the transit of secretory proteins through the Golgi complex have been reported (*15*), destruction of the Golgi complex by monensin may result in impairment of the interaction between tropoelastin and its receptor, leading to the instability of tropoelastin. In fact, it has been suggested that tropoelastin and the 67 kDa receptor are colocalized intracellularly, and that the two proteins are associated in the secretory pathway (*16*). It has been postulated that the receptor functions as a "molecular chaperone" by providing trafficking signals to direct the proper movement of the receptor-tropoelastin complex to the site of elastin fiber formation on the cell surface (*17*). The intracellular accumulation of tropoelastin on monensin treatment may cause the generation of tropoelastin free from the receptor leading to rapid degradation of tropoelastin.

The level of reduction of tropoelastin mRNA to one-fifth of the control level was not sufficient to explain the marked reduction of tropoelastin synthesis caused by monensin during period of 18-48 h to one-tenth of the control level. Therefore, the monensin-induced decrease in tropoelastin synthesis may be controlled through two different mechanisms, *i.e.* the reduction of tropoelastin mRNA and accelerated intracellular degradation of tropoelastin.

REFERENCES

1. Mollenhauer, H.H., Morré, D.J., and Rowe, L.D. (1990) Alteration of intracellular traffic by monensin; mechanism, specificity and relationship to toxicity. *Biochim. Biophys. Acta* **1031**, 225-246
2. Uchida, N., Smilowitz, H., and Tanzer, M.L. (1979) Monovalent ionophores inhibit secretion of procollagen and fibronectin from cultured human fibroblasts. *Proc. Natl. Acad. Sci. USA* **76**, 1868-1872
3. Uchida, N., Smilowitz, H., Ledger, P.W., and Tanzer, M.L. (1980) Kinetic studies of the intracellular transport of procollagen and fibronectin in human fibroblasts. *J. Biol. Chem.* **255**, 8638-8644
4. Frisch, S.M., Davidson, J.M., and Werb, Z. (1985) Blockage of tropoelastin secretion by monensin represses tropoelastin synthesis at a pretranslational level in rat smooth muscle cells. *Mol. Cell Biol.* **5**, 253-258
5. Tajiri, K., Uchida, N., and Tanzer, M.L. (1980) Undersulfated proteoglycans are secreted by cultured chondrocytes in the presence of the ionophore, monensin. *J. Biol. Chem.* **255**, 6036-6039
6. Yanagishita, M. and Hascall, V.C. (1985) Effects of monensin on the synthesis, transport, and intracellular degradation of proteoglycans in rat ovarian granulosa cells in culture. *J. Biol. Chem.* **260**, 5445-5455
7. Bumol, T.F. and Reisfeld, R.A. (1982) Biosynthesis and secretion of fibronectin in human melanoma cells. *J. Cell Biochem.* **21**, 129-140
8. Wollinsky, H. and Daly, M.M. (1970) A method for the isolation of intima-media samples from arteries. *Proc. Soc. Exp. Biol. Med.* **135**, 364-368
9. Oakes, B.W., Batty, A.C., Handley, C.J., and Sandberg, L.B. (1982) The synthesis of elastin, collagen, and glycosaminoglycans by high density primary cultures of neonatal rat aortic smooth muscle. An ultrastructural and biochemical study. *Eur. J. Cell Biol.* **27**, 34-46
10. Chomczynski, P. and Sacchi, N. (1987) Single step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162**, 156-159
11. Tokimitsu, I. and Tajima, S. (1994) Inhibition of elastin synthesis by high potassium salt is mediated by Ca^{2+} influx in cultured smooth muscle cells *in vitro*: Reciprocal effects of K^+ on elastin and collagen synthesis. *J. Biochem.* **115**, 536-539
12. Tokimitsu, I., Tajima, S., Nishikawa, T., Tajima, M., and Fukasawa, T. (1987) Sequence analysis of elastin cDNA from chick aorta and tissue specific transcription of the elastin gene in developing chick embryos. *Arch. Biochem. Biophys.* **256**, 455-461
13. Cleveland, D.W., Lopata, M.A., MacDonald, R.J., Cowan, N.J., Rutter, W.J., and Kirschner, M.W. (1980) Number and evolutionary conservation of α- and β-tubulin and cytoplasmic β- and γ-actin genes using specific cloned cDNA probes. *Cell* **20**, 95-105
14. Hayashi, A., Wachi, H., and Tajima, S. (1995) Presence of elastin-related 45-kDa fragment in culture medium: specific cleavage product of tropoelastin in vascular smooth muscle cell culture. *Biochim. Biophys. Acta* **1244**, 325-330
15. Lodish, H.F., Kong, N., Snider, M., and Strous, G.J.A.M. (1983) Hepatoma secretory proteins migrate from rough endoplasmic reticulum to Golgi at characteristic rates. *Nature* **304**, 80-83
16. Hinek, A. and Mecham, R.P. (1990) Characterization and functional properties of elastin receptor in *Elastin; Chemical and Biological Aspects* (Tamburro, A.M. and Davidson, J., eds.) pp. 369-381, Gelatina Congedo, Padova, Italy
17. Hinek, A. and Rabinovitch, M. (1994) 67-kD elastin-binding protein is a protective "companion" of extracellular insoluble elastin and intracellular tropoelastin. *J. Cell Biol.* **126**, 563-574