# Human Recombinant Vasostatin-1 May Interfere with Cell–Extracellular Matrix Interactions

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**ABSTRACT: Vasostatin-1 (VS-1), the N-terminal fragment derived from the cleavage of chromogranin A (CgA), has been shown to exert several biological activities on several tissues and organs. Recently, it has been reported that human recombinant VS-1 (STA-CGA$_{1-78}$) may alter myocardial contractility in eel, frog, and rat hearts. In this article we have explored if STA-CGA$_{1-78}$ can induce intracellular cascades interacting both with adhesion molecules and/or extracellular matrix (ECM), components, that is, involvement of the heat shock protein 90 (HSP90) and the endothelial NOS (eNOS), known to be implicated in signal transduction mechanisms affecting myocardial contractility. We used 3D cultured adult rat cardiomyocytes cultivated over fibronectin or fibroblasts or embedded in matrigel or collagen type I. Aurion-conjugated VS-1 (Au-STA-CGA$_{1-78}$) has been used to identify possible sites of interaction of this molecule with the cell membrane. We found that in our 3D culture, cell–ECM interactions played a crucial role in the cellular localization of HSP90 as well as in the expression of eNOS. VS-1 appeared to modulate cell–ECM interactions, thereby remarkably leading to a different cellular localization of HSP90. Moreover, Au-STA-CGA$_{1-78}$ was never detected inside the cell nor overlapping the plasma membrane, but nearby the outer side of the cardiomyocyte plasmalemma, at a particular distance, typical of integrins. On the whole, these data suggest that VS-1 does not have a classic receptor on the membrane but that integrins may represent a nonconventional VS-1 receptor modulating eNOS signaling pathway.**

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## INTRODUCTION

Chromogranin A (CgA) belongs to the family of chromogranins, which are secretory proteins localized into the electron-dense granules of several endocrine and neuroendocrine cells[1,2] and released into circulation. Peptides deriving from CgA have been detected in several tissues[3] and exhibited unusual biological properties. Among them, they have been identified two human vasostatins (VSs): $CgA_{1-76}$ (vasostatin-1; VS-1) and $CgA_{1-113}$ (vasostatin-2; VS-2). They can act as regulatory peptides in an autocrine, paracrine, or endocrine manner.[4]

Recently, it has been reported that a human recombinant VS-1 (STA-$CGA_{1-78}$) can decrease important parameters of myocardial inotropism, such as stroke volume (SV) and stroke work (SW), in eel[5] and frog[6] hearts, and left ventricular pressure (LVP) and rate pressure product (RPP) in the Langendoff-perfused rat heart.[7] Both in rat and eel hearts this negative inotropism seemed to be dependent from the presence of the endocardial endothelium (EE) and from the NO-cGMP signal transduction pathway, while in the frog heart it seemed independent.

The enzyme responsible for the production of most of the NO in myocardium is the endothelial nitric oxide synthase (eNOS). eNOS is usually localized in the plasma membrane caveolae of cardiomyocytes. *In vitro* inactive eNOS can associate with caveolin-3 and can be activated, following appropriate stimuli, through phosphorylation by Akt/PKB kinase, coupled to eNOS by heat shock protein 90 (HSP90) binding.[8–10]

In this article we used 3D-cultured adult rat cardiomyocytes to explore possible sites of interactions of STA-$CGA_{1-78}$, containing the VS-1 $CGA_{1-76}$ sequence, with the cell membrane or with extracellular components of myocardial extracellular matrix (ECM). Two proteins have been considered as markers of STA-$CGA_{1-78}$ action and aurion-conjugated STA-$CGA_{1-78}$ (Au-STA-$CGA_{1-78}$) has been used to identify putative sites of VS-1 interactions with components of either the cardiac cells or ECM.

The results show that the expression of HSP90 and eNOS proteins in 3D-cultured cardiomyocytes can be affected by STA-$CGA_{1-78}$ and that these phenomenon can be related to the ECM components used as culture substrates. Furthermore, Au-STA-$CGA_{1-78,}$ was always visible extracellularly nearby the plasma membrane.

## MATERIALS AND METHODS

Freshly dissociated cardiomyocytes, obtained from rat ventricles treated with 50 U/mL collagenase II were maintained in culture for no more than 10 days

in M-199 medium supplemented with 20% fetal calf serum (FCS), with antibiotics and antimycotics.

Four different coating substrates were used in the cultures: rat collagen type I; matrigel basement membrane (GFR); human plasma fibronectin; and a monolayer of cardiac fibroblasts obtained from the same myocardium. After 24 h from plating, cells were treated with STA-CGA$_{1-78}$ 5–10 μg/mL for 30 min. After treatment cells were fixed and used for immunofluorescence and electron microscopy. Recombinant Ser-Thr-Ala-hCGA$_{1-78}$ (STA-CGA$_{1-78}$) was obtained as previously described.[7,11]

For 3D-culture gels, aliquots of isolated cardiomyocytes were suspended in a collagen type I solution or in a matrigel solution. The gels were superfused with M-199 medium-20% FCS and placed in a cell culture incubator.

For transmission electron microscopy $5 \times 10^4$ cardiomyocytes were mixed with matrigel and placed into 24-well plate inserts. After STA-CGA$_{1-78}$ treatment, cells were fixed, dehydrated until ethanol 70% (v/v), and embedded into L.R.White resin. For experiments with Au-STA-CGA$_{1-78}$ (3- to 4-nm-diameter beads), one out of two inserts was treated with 5 μg/mL Au-STA-CgA$_{1-78}$ for 1 h, before fixation.

For immunofluorescence experiments methanol fixed cells were blocked and incubated with primary antibodies (1:50, anti-HSP90 α/β and anti-eNOS). Cells were then incubated with fluorescent secondary antibody (FITC-conjugated anti-mouse secondary antibody and FITC-conjugated anti-rabbit secondary antibody). Imaging was done on a Leica Laser Scannin Confocal Microscope (Leica Microsystems GmbH, Wetzlar, Germany).

For Western blotting analysis cardiac fibroblasts were lysated with RIPA lysis buffer containing proteases and phosphatases inhibitors. Total proteins were determined using the DC Protein Assay Kit (Biorad, Hercules, CA). The same amount of proteins was run on SDS-PAGE and transferred to PVDF membranes. Secondary antibody was detected using ECL.

## RESULTS

At electron microscopy mitochondria of isolated cardiomyocytes appeared intact and used as a control for cellular viability. In some cardiomyocytes myofibrils lost their organization. Cardiomyocytes cultured over fibronectin or a layer of cardiac fibroblasts, or embedded in matrigel or collagen type I were treated with 5 or 10 μg/mL STA-CGA$_{1-78}$ after 24 h from plating.

HSP90 α/β protein was localized only in small compartments inside the cell and its level did not change after treatment when cells were cultivated over fibronectin or embedded in collagen type I. The same protein was localized beneath the cardiomyocyte plasma membrane when cells were cultivated embedded in matrigel or over a layer of cardiac fibroblasts. After exposure to 10 μg/mL STA-CGA$_{1-78,}$ HSP90 α/β moved to small internal compartments

near the nucleus. eNOS enzyme expression in matrigel cultures varied after treatment with STA-CGA$_{1-78}$ at the concentrations of 5–10 μg/mL.

To study the possible binding sites of STA-CGA$_{1-78}$ to the cellular membrane, we used Au-STA-CGA$_{1-78}$, with 3- to 4 nm-diameter beads. Cardiomyocytes, plated inside a 50-μm-thick matrigel gel, were treated with 5 μg/mL Au-STA-CGA$_{1-78}$. In samples treated for electron microscopy, Au-STA-CGA$_{1-78}$ was visible outside the cell and nearby the outer side of the cardiomyocyte plasma membrane at a minimum distance in the range between 16 and 25 nm (FIG. 1). No molecules were found inside the cell or overlapping the plasma membrane.

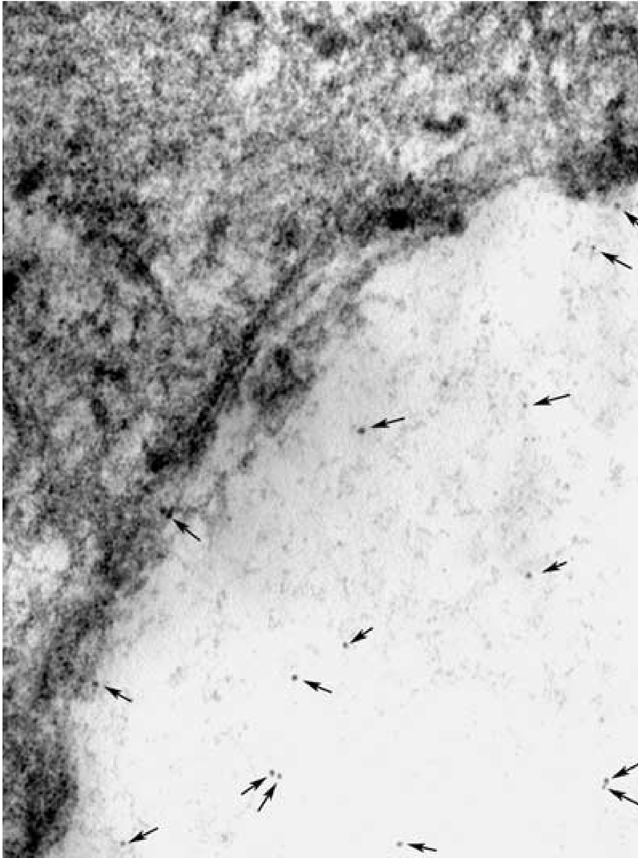

**FIGURE 1.** Transmission electron microscopy shows the position of 3- to 4-nm-aurion beads, representing Au-STA-CGA$_{1-78}$ molecules in 3D cultures of cardiomyocytes (*black arrows*).

## DISCUSSION

These results demonstrate for the first time that STA-$CGA_{1-78}$ affects HSP90 protein cellular localization and eNOS expression in cardiomyocytes cultured in matrigel three dimensionally. They also show that Au-STA-$CGA_{1-78}$ exclusively localizes outside the plasma membrane of cardiomyocytes.

Mitochondria integrity, HSP90 basal expression, and eNOS induction, apart from their cellular function, can be considered markers of cell viability. We used different substrates in the cultures: fibronectin, collagen type I, matrigel, and cardiac fibroblasts. An important finding of the present work is that HSP90 protein changes localization depending on the substrate used. In particular HSP90 was detected beneath the plasma membrane when cardiomyocytes were cultured on cardiac fibroblasts or in matrigel, suggesting the importance of cell–ECM molecular interactions in the subcellular compartmentalization of this protein.

Cardiomyocytes cultured in matrigel appeared to be the best system to study VS-1 effects also on eNOS expression. After exposure to 5–10 μg/mL STA-$CGA_{1-78}$ for 30 min, eNOS protein level increases perhaps for new protein production.

Even if HSP90 and eNOS did not show the same cellular localization, we think that their expressions are correlated, because it is already known in literature that eNOS activation is dependent on HSP90 caveolar localization.[12]

An interference with cell–ECM interactions has been postulated as a possible mechanism of action of STA-$CGA_{1-78}$ on cultured cardiomyocytes. To study this kind of interactions 3D cultures were treated with Au-STA-$CGA_{1-78}$. In our experiments, the aurion conjugated molecule was always found at a particular distance from the plasma membrane (between 16 and 25 nm). This distance is typical of interactions between cells and ECM proteins.

Our findings may lead to the working hypothesis that natural VS-1 may act *in vivo* interfering with cell–ECM interactions. However, neither a conventional receptor nor the action sites of VS-1 are known. Two putative domains of the human recombinant VS-1 could be taken into consideration for its binding either to the cell membrane or to the ECM components: an RGD sequence at residues 43-45[13] and a net positively charged domain at residues 47–70.[14] Anyway, the RGD site of CgA is not conserved among different species and its involvement in the regulation of cell–ECM interactions remains to be proved.

Another possibility is that VS-1-induced HSP90 localization and eNOS expression could result from VS-1 interaction with membrane phospholipids and consequent enhancement of membrane fluidity. However, a combination of the two mechanisms can be postulated.

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