Journal of Molecular and Cellular Cardiology 51 (2011) 777–780

Contents lists available at SciVerse ScienceDirect

Journal of Molecular and Cellular Cardiology

journal homepage: www.elsevier.com/locate/yjmcc

Original article

Dynamics of heat shock protein 60 in endothelial cells exposed to cigarette smoke extract

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ARTICLE INFO

Article history:
Received 7 March 2011
Received in revised form 27 June 2011
Accepted 4 July 2011
Available online 12 July 2011

Keywords:
Cigarette smoking
Heat shock protein 60
Atherosclerosis
Autoimmunity
Live cell imaging

ABSTRACT

Heat shock protein 60 (HSP60), expressed on the surface of endothelial cells (ECs) stressed by e.g. oxidized LDL or mechanical shear, was shown to function as an auto-antigen and thus as a pro-atherosclerotic molecule. The aim of this study was to determine whether cigarette smoke chemicals can lead to the activation of the "HSP60 pathway." It was also our aim to elucidate the dynamics of HSP60 from gene expression to endothelial surface expression and secretion. Here we show for the first time that the exposure of human umbilical vein endothelial cells (HUVECs) to cigarette smoke extract (CSE) results in an up-regulation of HSP60 mRNA. Live cell imaging analysis of a HSP60-EYFP fusion protein construct transfected into ECs revealed that mitochondrial structures collapse in response to CSE exposure. As a result, HSP60 is released from the mitochondria, transported to the cell surface, and released into the cell culture supernatant. Analysis of HSP60 in the sera of healthy young individuals exposed to secondhand smoke revealed significantly elevated levels of HSP60. Cigarette smoking is one of the most relevant risk factors for atherosclerosis. Herein, we provide evidence that cigarette smoke may initiate atherosclerosis in the sense of the "auto-immune hypothesis of atherosclerosis."

1. Introduction

It is generally accepted that atherosclerosis is an inflammatory disease of the arterial vessel wall [1,2]. Our previous observations suggest that inflammation is also an essential factor in the incipient stages of atherosclerosis [2]. ECs exposed to stress caused by classical atherosclerosis risk factors show surface expression of heat shock protein 60 (HSP60). Infections during childhood resulting in the formation of anti-microbial heat shock protein antibodies, and a cross-reactivity of these antibodies to human heat shock protein 60 (due to high sequence homologies) are the basis for an auto-immune reaction against ECs. It has been shown that atherosclerosis risk factors such as oxidized low-density lipoproteins and shear stress lead to the translocation of HSP60 to the EC surface, where it serves as an auto-antigen and signalling molecule [3–5]. Anti-HSP60 antibody-mediated cellular cytotoxicity has been observed, and in several human studies, anti-HSP60 antibody serum titres were correlated with atherosclerosis and other autoimmune diseases [2].

In a study of healthy young adults, cigarette smoking was found to be the most relevant risk factor for early atherosclerosis, indicating the impact of this risk factor in atherosclerosis initiation [6]. Experimental microarray-based studies demonstrated that cigarette smoke extract (CSE) provokes a massive heat shock response in ECs at the transcriptome level [7]. The effects of cigarette smoke chemicals on HSP60 at the cellular level (particularly the dynamics and surface expression) as well as the dependence of serum HSP60 levels on the smoking status in young individuals have not been studied to date. Based on previous results on other risk factors for early atherosclerosis, this study was designed to address the hypothesis that the risk factor smoking, too, may induce in stressed ECs an HSP60 response ranging from activation of transcription to the translocation of HSP60 from the mitochondria to the cytosol, and from there to the EC surface. It has previously been reported that soluble HSP60 activates macrophages [8]. In this study, we wanted to determine if soluble HSP60 also binds to macrophages. To ascertain the *in vivo* relevance of this investigation, we examined not only the EC culture supernatants but also serum samples from healthy young smokers, passive smokers (secondhand smokers) and non-smokers, for the presence of soluble HSP60.

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doi:10.1016/j.yjmcc.2011.07.003

2. Materials and methods

For details on Materials and methods, see the Online Supplement.

3. Results

3.1. HSP60 mRNA expression and dynamics of HSP60 protein in endothelial cells exposed to CSE

First, the effect of CSE on endothelial HSP60 mRNA expression was analyzed after different incubation times. Fig. 1A shows that an up-regulation of hspd1 mRNA occurs as early as 3 h after exposure to 8% CSE and peaks at 7 h. The transient character of this upregulation is indicated by the drop in the levels of HSP60 mRNA after 24 h.

To define the dynamics of HSP60 in ECs exposed to CSE, live cell imaging analyses were performed. Figs. 1B–E and the video file (Online Supplement) revealed that CSE leads to significant changes in mitochondrial morphology and alters cellular HSP60 localization (CSE was added at time point 0, live cell imaging was conducted for 180 min). The major findings were (i) disruption of the filamentous phenotype of mitochondria (for comparison also see Fig. 1H) 60 min after addition of CSE (indicated by a white arrow in Fig. 1C), and (ii) partial disintegration of mitochondria, leading to HSP60 release from mitochondria and its cytosolic appearance (orange arrows in Fig. 1E). In order to test whether, in addition to causing a release of HSP60 from the mitochondria into the cytosol, CSE also causes translocation of HSP60 to the cell surface, ECs were incubated with 8% CSE for 24 h, followed by intra-cellular and membrane immuno-staining for HSP60. Figs. 1F–K show that, in contrast to the intracellular filamentous staining pattern in the controls, CSE-treated cells show a membrane-associated distribution of HSP60 (black arrows indicate surface HSP60).

3.2. HSP60 is released into the culture supernatant in response to CSE treatment of EC

To address the question whether surface HSP60 is released into the culture supernatant, an ELISA-based quantification of soluble HSP60 in the supernatant of ECs treated with different concentrations of CSE

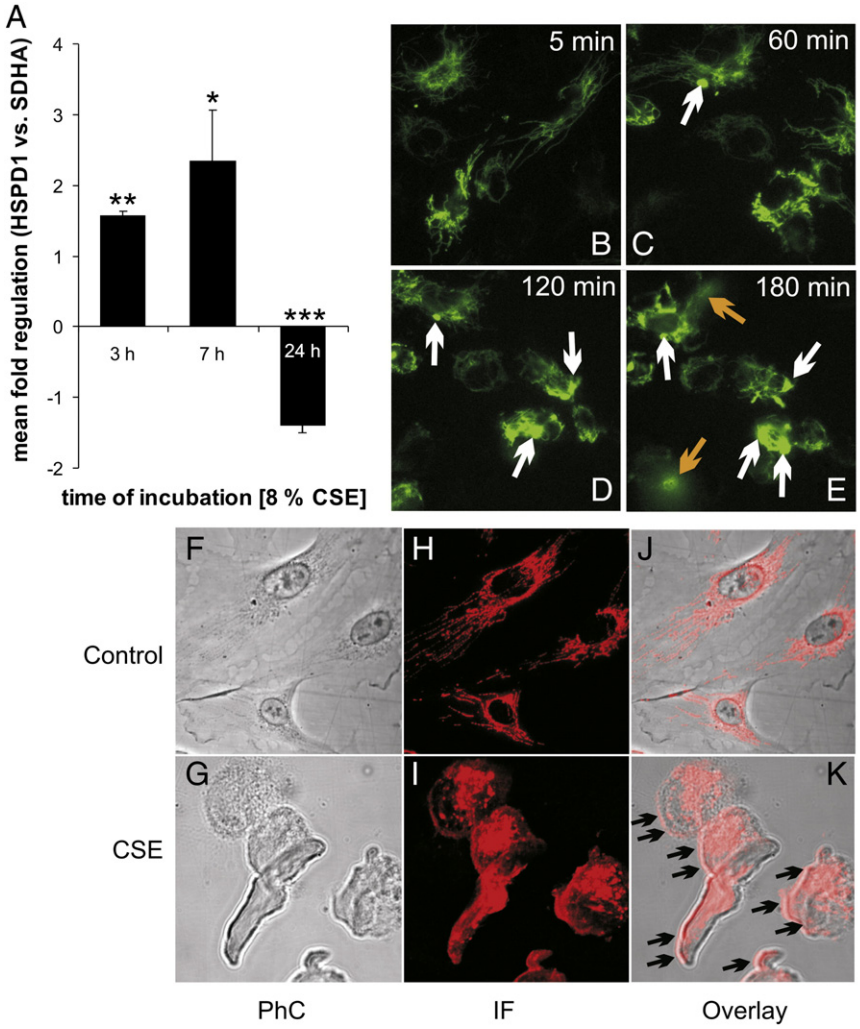

Fig. 1. CSE induces the upregulation of HSP60 mRNA, causes HSP60 release from mitochondria, and leads to HSP60 surface expression. A. Real-time PCR analysis of hspd1 mRNA in response to exposure of HUVECs to 8% CSE over different time periods. Images B to E show the effects of treating HUVECs with 16% CSE over different time periods on HSP60-EYFP localisation. White arrows indicate contracted mitochondria; orange arrows indicate cells where HSP60-EYFP was released from mitochondria. Images F, H, and J show control HUVECs; images G, I, and K show HUVECs treated with 8% CSE for 24 hours. Images F, G: Phase contrast analysis (PhC); images H, I: HSP60 immunofluorescence analysis; images J, and I: overlay of PhC and IF. Black arrows indicate a plasma membrane-associated localisation of HSP60. All experiments were repeated three times. Shown are either representative images or mean values of a representative experiment performed in duplicates ±SD. Asterisks indicate significant differences (determined by ANOVA; Posthoc Bonferroni) of the groups compared to untreated control cells. (*$p<0.05$; **$p<0.01$; ***$p<0.001$).