Heat shock protein 60 induces inflammatory mediators in mouse adipocytes

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Abstract Adipocytes represent an important cellular source of inflammatory mediators. However, the signals for the induction of proinflammatory adipocyte activities are largely unknown. Here, we demonstrate that heat shock protein (Hsp) 60, a potent stimulator of innate immunity, induces the release of the inflammatory mediators interleukin-6, CXCL1 and monocyte chemoattractant protein-1 in a time- and concentration-dependent manner from cells of the adipocyte line 3T3-L1 and from adipocytes of obese mice. These results identify Hsp60 as an important regulator of adipocyte functions which contribute to the development of inflammatory processes as observed in diabetes and diabetes-associated complications.

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1. Introduction

Adipose tissue, for a long time mainly characterized by its fat storing capacity, has recently been recognized as the origin of a variety of mediators with potent inflammatory effects [1]. Meanwhile cells of the adipocyte fraction have been identified as an important source of numerous inflammatory mediators including interleukin-6 (IL-6), IL-8 and monocyte chemoattractant protein-1 (MCP-1) [2,3]. Further studies now provide increasing evidence that adipocyte-derived cytokines and chemokines sustain systemic low grade inflammation thereby promoting the development of metabolic disorders, in particular type 2 diabetes, and associated cardiovascular complications [1,4]. More detailed investigations on the functional activities of adipocytes revealed that this cell type shares many basic features with innate immune cells, such as macrophages [5]. However, until now the physiological signals triggering the formation of inflammatory mediators in adipocytes remain largely unknown.

Meanwhile it could be demonstrated that highly efficient stress signalling for macrophages, is provided by heat shock protein (Hsp) 60 [6,7], a prominent member of the HSP family [8], that exerts its stimulatory activity by specifically bound bacterial lipopolysaccharide (LPS) [9]. Although these findings in innate immune cells qualify Hsp60 as potential signal for the induction of proinflammatory mediators from adipocytes, currently no data on the effects of Hsp60 on adipocyte activities are available.

Therefore, the aim of our study was to investigate the effect of Hsp60 on the induction of proinflammatory mediators in adipocytes. By the use of the murine adipocyte line 3T3-L1 [10] and adipocytes of the New Zealand obese (NZO) mouse, a model of human adiposity and the metabolic syndrome [11], we could demonstrate for the first time that adipocytes are able to respond to Hsp60. Hsp60 dose-dependently induced the release of IL-6, of KC (CXCL-1), the murine homologue of human IL-8, and of MCP-1, indicating that inflammatory adipocyte functions are under control of stress proteins as potent endogenous danger signals.

2. Materials and methods

2.1. Mice and 3T3-L1 cells

NZO mice were obtained from the breeding colony at the German Diabetes Center. The mouse preadipocyte cell line 3T3-L1 was purchased from ATCC, Manassas, VA, and was cultivated in DMEM with 4.5 g/l glucose (PAA Laboratories GmbH, Coellbe, Germany) supplemented with 10% (v/v) calf serum (PAA Laboratories GmbH), antibiotic–antimycotic solution (Invitrogen GmbH, Karlsruhe, Germany) and 1 mM Na-Pyruvat (PAA Laboratories GmbH) (culture medium).

2.2. Isolation of murine adipocytes

Adipocytes were isolated from visceral adipose tissue of 6–9 months old, normoglycemic, female NZO mice as described previously [12]. The resulting cells were cultivated and expanded in culture medium.

2.3. Determination of inflammatory mediators

3T3-L1 cells and primary preadipocytes (1 × 10⁵/500 µl per well of a 48 well culture plate) were exposed to LPS (Escherichia coli O26:B6, Sigma–Aldrich, Steinheim, Germany), to recombinant human Hsp60 (LPS contents 1.2 pg/µg protein) (Stressgen, Victoria, BC, Canada) or to combinations of both reagents. After different incubation periods the concentrations of accumulated IL-6, KC and MCP-1 in the supernatants were determined by ELISA (BD Biosciences, Heidelberg, Germany; R&D Systems GmbH, Wiesbaden, Germany).

2.4. Oil Red O staining

Oil Red O staining was performed according to a previously described method [13].

Abbreviations: Hsp60, heat shock protein 60; IL-6, interleukin-6; KC, mouse chemokine CXCL1; MCP-1, monocyte chemoattractant protein-1; NZO, New Zealand obese; Pref-1, preadipocyte factor-1; LPS, lipopolysaccharide

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2.5. FACS analysis

To prove the purity of the primary adipocyte preparations and to exclude macrophage-contaminations, the cells were stained with an allopurinol-conjugated rat anti-mouse CD11b monoclonal antibody (BD Biosciences). The macrophage line J774A.1 was used as positive control for CD11b expression. The FITC-labelled rat anti-mouse preadipocyte factor-1 (Pref-1) monoclonal antibody (MBL, Woburn, MA, USA) was used to determine the adipocyte maturation state. Analyses were performed with a FACS Calibur flow cytometer (BD Biosciences).

2.6. Statistical analysis

Data were expressed as mean values ± S.D. Statistical analysis was performed using the Student's t-test. Differences were considered statistically significant with P < 0.05.

3. Results

3.1. Induction of the cytokine IL-6 in 3T3-L1 mouse preadipocytes by LPS and Hsp60

The stress signals LPS and Hsp60 induce the release of IL-6 from 3T3-L1 cells (Fig. 1A). LPS caused a dose-dependent increase of IL-6 concentrations up to 1.2 ± 0.1 ng/ml in the presence of 1000 ng/ml LPS (24 h). Exposure to rising Hsp60 concentrations resulted in an increase of IL-6 release up to 0.46 ± 0.04 ng/ml at 20 μg/ml Hsp60.

IL-6 accumulation induced by 10 and 1000 ng/ml LPS was detectable already after 3 h and reached its maximum (0.9–1.4 ng/ml) after 24–48 h (Fig. 1B). Exposure to Hsp60 for 3 h induced significant IL-6 amounts (Fig. 1C) which remained at an elevated level of about 0.2 ng/ml (10 μg/ml Hsp60) or declined after a peak (0.5 ± 0.1 ng/ml) at 24 h (20 μg/ml Hsp60).

3.2. Induction of the chemokines KC and MCP-1 in 3T3-L1 mouse preadipocytes by LPS and Hsp60

LPS- or Hsp60-exposure of 3T3-L1 cells dose-dependently induced the release of KC up to 36.6 ± 1.0 ng/ml and 31.1 ± 2.2 ng/ml, respectively (Fig. 2A). KC levels rapidly increased over the observation period and reached 44.9 ± 0.3 ng/ml in LPS-exposed cells (Fig. 2B) and 41.5 ± 3.7 ng/ml in Hsp60-treated cells (Fig. 2C).

Fig. 1. LPS- and Hsp60-induced IL-6 release from 3T3-L1 adipocytes. 3T3-L1 cells were incubated in the absence (medium) or presence of different concentrations of LPS (open bars) or Hsp60 (solid bars) for 24 h (A). For kinetic studies the cells were exposed to LPS (B) or Hsp60 (C) as indicated for 0–72 h. IL-6 concentrations were determined by ELISA. Data show means ± S.D. of three determinations. **P < 0.01; ***P < 0.001 compared to corresponding medium control.

Fig. 2. LPS- and Hsp60-induced KC formation from 3T3-L1 cells. 3T3-L1 cells were incubated in the absence (medium) or presence of different concentrations of LPS (open bars) or Hsp60 (solid bars) for 24 h (A). For kinetic studies the cells were exposed to LPS (B) or Hsp60 (C) as indicated for 0–48 h. KC concentrations were quantified by ELISA. Data show means ± S.D. of three determinations. **P < 0.01; ***P < 0.001 compared to corresponding medium control.
A dose-dependent increase of MCP-1 concentrations up to 38.7 ± 0.1 ng/ml and 42.4 ± 5.0 ng/ml was observed in 3T3-L1 cultures treated for 24 h with LPS and Hsp60, respectively (Fig. 3A). MCP-1 levels increased to 36.2 ± 2.6 ng/ml in the presence of 1000 ng/ml LPS (Fig. 3B) and to 64.6 ± 7.2 ng/ml in the presence of 20 µg/ml Hsp60 (Fig. 3C).

3.3. Synergistic effects of Hsp60 and LPS on the induction of inflammatory mediators by 3T3-L1 mouse preadipocytes

To identify potential synergistic effects of Hsp60 and LPS, 3T3-L1 cells were exposed to combinations of the reagents in suboptimal stimulatory concentrations and after 24 h the levels of IL-6, KC and MCP-1 (Fig. 4) were determined. The results show that the stimulatory activity of combinations of Hsp60 and LPS is generally above the activity of the individual agents, indicative for their additive effect. Significant synergistic effects on the production of IL-6 and KC were observed when 10 µg/ml Hsp60 was combined with 1 or 3 ng/ml LPS (Fig. 4A and B), whereas combinations of 10 µg/ml Hsp60 and LPS concentrations of 3 or 10 ng/ml showed significant synergistic effects on the induction of MCP-1 (Fig. 4C).

3.4. LPS and Hsp60 induce the release of inflammatory mediators from adipocytes of NZO mice

To determine whether LPS and Hsp60 are also able to stimulate the inflammatory activity of primary adipocytes, we analysed the responsiveness of adipocytes from NZO mice to these stress signals.

FACS analysis of the isolated adipocyte population demonstrated the lack of the macrophage specific marker CD11b in >99% of the cells thereby proving the absence of contaminating macrophages. Staining with the fat specific dye Oil Red O revealed a typical lipid droplet staining pattern in >99.2% of the cells proving their adipocyte identity and further confirming the purity of the adipocyte population. FACS-based detection of the adipocyte-specific maturation marker Pref-1
identified the vast majority of the cells (75 ± 18%) as immature (pre-)adipocytes. Exposure of the cells to LPS or Hsp60 for 24 h resulted in a dose-dependent release of IL-6, KC and MCP-1. Stimulation with 10 ng/ml LPS was sufficient to induce maximum levels of IL-6 (1.6 ± 0.1 ng/ml), KC (9.8 ± 0.1 ng/ml) and MCP-1 (47.0 ± 1.5 ng/ml) (Fig. 5A–C). After Hsp60 stimulation maximum release of IL-6 (1.1 ± 0.2 ng/ml) (Fig. 5A) and MCP-1 (39.5 ± 0.1 ng/ml) (Fig. 5C) was observed at a concentration of 20 µg/ml of the stress protein, whereas the induction of maximum KC levels (9.4 ± 0.1 ng/ml) was already reached at an Hsp60 concentration of 10 µg/ml (Fig. 5B). Interestingly, both LPS and Hsp60 failed to induce the release of TNF-α from 3T3-L1 cells and NZO-derived adipocytes.

4. Discussion

Adipocytes have been recognized as integral components of the complex network of tightly interacting metabolic and immunological processes [1]. In view of their central role in obesity and related diseases, adipocyte activities involved in metabolic control have long been in the focus of interest, whereas at present only limited information is available on adipocyte functions relevant for the control of immunological processes. Although recent findings demonstrate a strong immunomodulatory potential of members of the HSP family [6,14], the possible effects of Hsp on adipocyte functions are unknown.

In this study, we now examined the effect of Hsp60, a prominent member of the HSP family and a highly potent endogenous stress signal, on the induction of inflammatory mediators from mouse preadipocytes. For our experiments, the adipocytes were exposed to Hsp60 concentrations covering a range from physiological serum levels in rodents (below 10 µg/ml) up to a concentration of 20 µg/ml, reflecting the elevated Hsp60 serum levels found under pathological conditions [15,16]. The studies were performed with cells of the adipocyte line 3T3-L1 complemented by investigations on primary adipocytes isolated from the visceral fat depot of the NZO mouse [11]. Control experiments confirmed the purity of the NZO mouse-derived adipocyte population and excluded the presence of contaminating cells, particularly macrophages which possess functional properties similar to adipocytes [5] and exhibit pronounced responsiveness to Hsp60 [7]. Both, 3T3-L1 cells and NZO mouse adipocytes showed significantly increased release of the cytokine IL-6 and of the chemokines KC and MCP-1 in response to LPS, thus confirming the high adipocyte-stimulating capacity of this microbial stress signal [3].

In comparative approaches, we were able to demonstrate for the first time that Hsp60 induces the release of the inflammatory mediators IL-6, KC and MCP-1 from 3T3-L1 cells as well as from primary NZO mouse-derived adipocytes. In both cell types the Hsp60-induced release of the mediators was strictly dose dependent. Both cell types showed a strict dose-dependence of the Hsp60-induced release of inflammatory mediators.

In an attempt to mimic inflammatory conditions generally characterized by the simultaneous appearance of different, potentially synergizing stress signals [17], we exposed 3T3-L1 cells to combinations of Hsp60 and LPS. Concurring with our previous findings on murine macrophages we observed additive stimulatory effects when Hsp60 was acting on 3T3-L1 cells in combination with LPS [9]. Treatment of the cells with mixtures of the agents at suboptimal stimulatory concentrations resulted in differential stimulatory effects dependent on the Hsp60-LPS ratio acting on the cells. The observed differential effects of the Hsp60-LPS combinations on the induction of the investigated inflammatory mediators most likely reflect the specific and highly complex interactions between the two stress signals that mutually affect their stimulatory capacities [9].

The striking similarity in the reactivity of cells of the adipocyte line and of the primary mouse adipocytes indicates that Hsp60 responsiveness represents a basic property of murine pre-adipocytes. The failure of Hsp60 to induce the release of TNF-α concurs with previous findings [3] and implicates that Hsp60-exposure does not cause a general, unspecific stimulation of adipocyte secretory activity but leads to the activation of selected pathways resulting in the formation of a distinct pattern of mediators. Our findings further implicate the presence of a yet undefined Hsp60 receptor on adipocytes. Since adipocytes in many instances resemble innate immune cells.
it might be speculated that the Hsp60 receptor on adipocytes shares basic features with the Hsp60 receptor on macrophages, a dominant population of antigen-presenting cells. The results of our previous studies on Hsp60-macrophage interactions suggest that the Hsp60 receptor complex comprises several functional components including the toll-like receptor 4 (TLR-4) as an indispensable structure for the mediation of the stimulatory Hsp60 effects [18,19]. The recent identification of functionally active TLR4 on adipocytes [20] implicates a role of this transmembrane protein also in Hsp60-adipocyte interactions.

Although mature adipocytes constitute by far the largest cell population of adipose tissue our present study focuses on pre-adipocytes since their functional activities largely resemble those of immunocompetent cells of the innate immune system [5]. Pre-adipocytes show phagocytic and microbicidal activity and, when compared to mature adipocytes, they exhibit a strongly increased responsiveness to stress signals of microbial origin [21].

Our observations with cultivated adipocytes could be of relevance for the understanding of the inflammatory processes associated with the development of metabolic disorders including obesity, diabetes and cardiovascular complications. It might be speculated that the systemically increased Hsp60 levels observed in these disease states [22,23] cause (chronic) adipocyte-stimulation. Another origin of adipocyte stimulating Hsp60 might be the adipose tissue itself, which is known to be a source of inflammatory activities [24] that could result in the local accumulation of stress signals. On the other hand, it is conceivable to assume that the Hsp60-induced release of inflammatory adipocyte mediators promotes the attraction and activation of immune cells in the adipose tissue [25] and contributes to the systemic elevation of pro-inflammatory mediators described in individuals with impaired glucose metabolism [26]. In the context of our present findings in murine adipocytes these considerations warrant further investigations with human adipocytes preferably isolated from the visceral fat depot, which is regarded as a major source of inflammatory mediators contributing to the pathogenesis of metabolic disorders and associated complications [27].

Taken together, this study demonstrates that adipocytes are able to respond to the endogenous danger signal Hsp60 by the release of inflammatory mediators, thereby sharing a basic feature with innate immune cells. Our findings implicate an important role of Hsp60 in the control of adipocyte activities that may contribute to the development of diabetes-associated inflammatory processes.

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