Albumin inhibits adipogenesis and stimulates cytokine release from human adipocytes

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Schlesinger, Janet B., Vanessa van Harmelen, Catherine E. Alberti-Huber, and Hans Hauner. Albumin inhibits adipogenesis and stimulates cytokine release from human adipocytes. Am J Physiol Cell Physiol 291: C27–C33, 2006.—Bovine serum albumin (BSA) is commonly used in adipocyte experiments as a binding protein for fat-soluble substances. Therefore, it is of interest to investigate whether BSA per se is influencing the functioning of human adipocytes in vitro. In the present study, we investigated the potential of BSA to affect the proliferation and differentiation capacity of human preadipocytes. BSA was found to inhibit adipose differentiation in a dose-dependent manner (being significant at concentrations of 2.5 μM), whereas proliferation was not affected. We further investigated the effect of BSA on the secretory function of adipocytes focusing on the release of selected cytokines. Preadipocytes and freshly isolated adipocytes incubated with BSA secreted significantly higher amounts of IL-6, -8, and -10, and TNF-α compared with cells incubated without BSA. The effects on cytokine secretion seemed to reside at the level of gene expression because BSA increased TNF-α and IL-6 mRNA in a dose-dependent manner. The results of the present study indicate that the presence of BSA in the culture medium has considerable effects on adipocyte function in vitro. These effects should be carefully considered for in vitro studies of adipose tissue.

MATERIALS AND METHODS

Subjects. Human adipose tissue was obtained from the subcutaneous or omental adipose tissue of 20 subjects undergoing either elective open abdominal surgery (e.g., herniectomy or sigma resection; n = 11) or abdominal plastic surgery (n = 9). No selection was made based on body mass index, age, or gender criteria. Informed consent was obtained from the subjects before the surgical procedure. The study protocol was approved by the ethical committee of the Heinrich-Heine-University, Düsseldorf, Germany. The adipose tissue samples (5–20 g) were immediately transported to the laboratory after being removed.

BSA. Different batches of BSA were used in the experiments. These were purchased from Sigma-Aldrich (Taufkirchen, Germany): 1) BSA standard grade; 2) BSA essentially fatty acid free, ≥96%; 3) BSA highly purified, essentially fatty acid free, and essentially globulin free, minimum 99%; and 4) BSA low endotoxin.

Preadipocyte isolation from human adipose tissue. Preadipocytes were isolated and cultured as described in detail elsewhere (7). In brief, the adipose tissue specimens were dissected from fibrous material and visible blood vessels, weighed, minced into small pieces, and digested in PBS containing 20 mg/ml BSA and 200 U/ml collagenase (Serva, Heidelberg, Germany) for 90 min at 37°C. The completely disaggregated tissue was centrifuged for 10 min at 200 g, and the pellet of stromal cells (i.e., preadipocytes) was resuspended in an erythrocyte lysis buffer (consisting of 0.154 mol/l NH₄Cl, 10 mmol/l K₂HPO₄, and 0.1 mM EDTA, pH 7.3) to remove contaminating red blood cells. The cell suspension was filtered through a polypropylene mesh (pore size 150 μm) to remove any remaining tissue. The cell suspension was then centrifuged for 10 min at 200 g. The cell pellet was washed in DMEM/F-12 medium, filtered (pore size 70 μM), centrifuged again, and resuspended in DMEM/F-12 medium. The total cell number was then determined microscopically with the use of a Neubauer chamber and trypan blue dye. The cells were seeded in 12-well plates at a density of 50,000 cells/cm² and incubated in DMEM/F-12 medium containing 50 μg/ml gentamycin and 10% fetal calf serum (Biochrom, Berlin, Germany) for 20 h at 37°C in 5% CO₂ to become attached. The cells were then washed twice with PBS, and differentiation was started, using DMEM/F-12 (50:50, vol:vol) with 10 μg/ml transferrin, 66 nmol/l insulin, 200 pmol/l T₃, and 100 nmol/l cortisol, 500 μmol/l IBMX, and 1 μg/ml
troglitazone. IBMX and troglitazone were added to the medium only during the first 3 days. The medium was renewed every 2 to 3 days. Isolation of mature adipocytes. Adipose tissue was dissected from fibrous material and incubated in Krebs-Ringer phosphate buffer (pH 7.4) containing 100 U/ml collagenase and 40 mg/ml BSA (standard grade) in a shaking bath at 37°C for 1 h. The adipocytes were filtered through a polycarbonate mesh (pore size 250 μm) and washed three times in Krebs-Ringer phosphate buffer with 0.1% BSA. The cells were incubated in DMEM/F-12 containing BSA at a concentration of 38, 110, and 300 μM, respectively, for 24 h at 37°C in 5% CO₂.

Assessment of adipocyte differentiation. α-Glycerophosphate dehydrogenase (GPDH) activity was measured as an index of adipose differentiation. Cells were washed with PBS and harvested in pre-chilled 50 mmol/l Tris-HCl buffer containing 1 mmol/l EDTA and 1 mmol/l mercaptoethanol. After sonication, aliquots of the cell extracts were added to an assay mixture containing 100 mmol/l triethanolamine-HCl buffer (pH 7.5), 2.5 mmol/l EDTA, 0.12 mmol/l NADH and 0.1 mmol/l mercaptoethanol and GPDH activity was measured spectrophotometrically at 340 nm. The reactions were started by adding 90 mmol/l dihydroxyacetone phosphate. GPDH was expressed as mU/mg total protein. The protein concentration in the cell extracts was measured according to a modification of the method described by Lowry et al. (9), using a precipitation step with 6% (vol/vol) trichloroacetic acid to avoid interference with lipids (10). BSA was used as a protein standard.

Assessment of adipocyte proliferation. Cell proliferation was assessed by counting the cell number in the growing cultures at 24-h intervals until day 8. After day 8, most of the cultures were confluent and contact inhibition occurred. The cell number of six randomly selected areas (each 1 mm²) in two wells was counted under the microscope (i.e., 12 counts on a single sample). One person was involved in the counting of the cell number.

Measurement of lactate dehydrogenase activity. Lactate dehydrogenase (LDH) release in the incubation medium was measured as an index for cytotoxicity. Aliquots of the incubation medium were added to an assay mixture containing (in mM) 81 Tris, 203 NaCl, and 0.24 NADH, pH 7.2. The reactions were started by the addition of 1.55 mM p-nitrophenol, and the breakdown of NADH was measured spectrophotometrically at 340 nm at 30°C for 30 min.

Measurement of cytokine release. The release of IL-2, -4, -6, -8, and -10, granulocyte macrophage-colony-stimulating factor (GM-CSF), IFN-γ, and TNF-α was measured simultaneously in the incubation medium with the use of a protein array system (Bioplex; Bio-Rad, Munich, Germany), according to the instructions of the manufacturer. The Bioplex Protein Array system is a novel assay that combines the principle of a sandwich immunoassay with the Luminex fluorescent bead-based technology (3).

Measurement of gene expression of IL-6, TNF-α, and GPDH. Total RNA was isolated from differentiated adipocytes using a silica gel-based membrane method (Machery-Nagel, Düren, Germany) according to the manufacturer’s instructions. Total RNA (0.1 μg) was reversely transcribed to cDNA to a final volume of 20 μl using the iScript cDNA synthesis kit (Bio-Rad).

To quantify the expression of IL-6, TNF-α, and GPDH, we applied TaqMan PCR using the ABI Prism 7000 sequence detection system (Applied Biosystems, Darmstadt, Germany). The reaction contained 1× TaqMan universal PCR master mix, 0.5 μM of forward and reverse primers and 0.25 μM of the TaqMan probes, which were all commercially available from Qiagen (Hilden, Germany; catalog no. 241041, 241033, and 241011). Thermal cycling proceeded with 40 cycles (PCR initial activation step: 2 min, 50°C; 10 min, 95°C, 40 cycles of 15 s 95°C, and 1 min 60°C). The expression of GPDH was assumed not to be influenced by BSA and therefore was used as a housekeeping gene. IL-6 and TNF-α mRNA levels were expressed after correction for GPDH.

Measurement of endotoxin in the incubation medium. The concentration of endotoxin in the media with and without different kinds of BSA was quantified by a chromogenic endotoxin test, a limulus amebocyte lysate assay, performed by the Cambrex Bioproducts LAL Testing Service (Verviers, Belgium). All determinations were performed in duplicate.

Statistical analysis. Data are expressed as means ± SD. For statistical comparisons, Student’s paired t-test was used. P < 0.05 was considered statistically significant.

RESULTS

Effect of BSA on the differentiation of preadipocytes into adipocytes. In five experiments, preadipocytes were isolated from human adipose tissue and differentiated in an adipogenic medium in the presence or absence of various concentrations of BSA (0.025, 0.25, 2.5, 25, 110, and 250 μM). At day 16, GPDH activity was measured as an index of adipose differentiation. Figure 1 shows that the presence of BSA in the differentiation medium reduced GPDH activity. Starting at 2.5 μM, the higher concentrations of BSA inhibited GPDH activity significantly (P < 0.05) by up to 55%.

Effect of BSA on preadipocyte proliferation. In three experiments, human preadipocytes were incubated in a medium containing DMEM/F-12 and 2.5% FBS, to which BSA was added in concentrations of 0.25, 2.5, 25, 110, or 250 μM. The concentration of 2.5 μM promoted proliferation compared with control from day 5 to day 7. At day 8, this effect could not be seen for the latter-mentioned concentration, but for concentrations of 25, 110, and 250 μM BSA in the media (data not shown).

Effect of BSA on the release of cytokines from differentiated preadipocytes. After 16 days of adipose differentiation, the cells were incubated for 24 h with various concentrations of BSA and the release of IL-2, -4, -6, -8, and -10, GM-CSF, IFN-γ, and TNF-α was measured in the incubation medium. In the absence of BSA in the incubation medium, only IL-6, IL-8, and IL-10 were easily detectable in the incubation medium. In the presence of 0.25 μM BSA, the release of IL-6, IL-8, GM-CSF, and TNF-α was significantly increased (P < 0.05)
and the release of these four cytokines was further stimulated at a concentration of 2.5 μM (P < 0.05). IL-10 and IFN-γ were significantly increased by BSA at a concentration of 2.5 μM (P < 0.05), whereas there was a tendency toward an increase for IL-4 at this BSA concentration (P = 0.06). IL-2 was hardly detectable in the medium but seemed to be influenced by BSA. However, the increase of IL-2 was not significant at any concentration (Fig. 2).

In two experiments, differentiated preadipocytes were incubated in the absence and presence of 2.5 μM BSA for 6, 24, and 48 h, respectively. Interestingly, with BSA, the levels of IL-10 and TNF-α were the highest after 6 h, whereafter they decreased constantly after 24 and 48 h. On the other hand, there was a constant increase of IL-4, IL-6, IL-8, GM-CSF, and IFN-γ after 6, 24, and 48 h in the presence of BSA (data not shown).

**Effect of BSA on mRNA levels of IL-6 and TNF-α in differentiated preadipocytes.** To examine whether the effects of BSA on cytokine release were associated with changes of gene expression, we measured mRNA levels of IL-6 and TNF-α in the absence and presence of BSA by quantitative RT-PCR. Differentiated adipocytes were incubated for 24 h

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**Fig. 2.** Effect of BSA on cytokine release from in vitro differentiated human preadipocytes. Human preadipocytes were differentiated until day 16 using a standardized adipogenic medium. The cells were then incubated without and with increasing concentrations of FFA-free BSA for 24 h. Subsequently, the concentration of selected cytokines in the incubation medium was measured using the Bioplex Protein Array system. The results are expressed as means ± SD (n = 7) and were tested for significance using a paired t-test (*P ≤ 0.05; **P ≤ 0.005; ***P ≤ 0.0005; #P = 0.0627). GM-CSF, granulocyte-macrophage-colony-stimulating factor.
without and with various concentrations of BSA in the incubation medium. As shown in Fig. 3, BSA significantly stimulated IL-6 mRNA expression in a dose-dependent manner. For the mRNA expression of TNF-α, the same trend was detectable. Also, with other batches of BSA (BSA essentially fatty acid free, BSA highly purified, BSA low endotoxin), a significant increase of IL-6 mRNA was observed (data not shown).

On day 16, differentiated preadipocytes were incubated in the absence or presence of 0.25 μM or 2.5 μM BSA for 6, 24, 48 h, respectively. As shown in Fig. 4, the mRNA levels of IL-6 and TNF-α were the highest after 6 h and then decreased significantly (P < 0.01) after 24 and 48 h.

Effect of BSA on freshly isolated adipocytes. In further experiments, the effect of BSA on freshly isolated adipocytes was studied. Adipocytes were isolated from adipose tissue and directly incubated in the presence or absence of two concentrations of BSA (38, 110, or 300 μM) for 24 h. IL-2, IL-4, GM-CSF, and IFN-γ were not detectable, neither in the absence nor in the presence of any concentration of BSA (data not shown). IL-6, -8, -10, and TNF-α levels seem to have increased in the presence of BSA (Fig. 5).

The concentration of 300 μM increased IL-6 secretion significantly and tended to increase the secretion of IL-8 and TNF-α. A dose-dependent increase by increasing concentration could only be seen for IL-8, but this was not significant.

Effect of BSA on LDH release. We also tested whether BSA has cytotoxic effects on adipocytes by measuring LDH release from the cells after incubation with BSA. For these experiments, preadipocytes were first differentiated in a normal adipogenic medium. Then, on day 16, the cells were incubated for 24 h with various concentrations of BSA. Figure 6 shows that BSA did not affect LDH release significantly up to a concentration of 2.5 μM. At a concentration of 300 μM, however, LDH release was significantly increased.

LDH levels were also measured in the medium of adipocytes in suspension culture. Enzyme activities were not affected by BSA up to a concentration of 300 μM (data not shown).

Endotoxin in incubation media. Commercially available albumin might be contaminated by small traces of endotoxin. To exclude that the results obtained were due to contaminating endotoxin, we determined the concentrations of endotoxin in the incubation media containing 2.5 μM BSA. As shown in Table 1, the endotoxin levels were <0.5 ng/ml.

**DISCUSSION**

The goal of the present study was to test whether BSA is modulating adipocyte differentiation and secretory function in vitro. We found that preadipocytes in the presence of BSA differentiated less well than those in the absence of BSA. The inhibitory effect started at a rather low concentration (2.5 μM, −0.02% wt/vol BSA) and was concentration dependent. Also, the secretory function of the adipocytes was influenced by BSA. Both differentiated preadipocytes and freshly isolated adipocytes increased their release of IL-6, -8, -10, and TNF-α, whereas differentiated preadipocytes also started to secrete a measurable amount of GM-CSF and IFN-γ when incubated with BSA. These results indicate that BSA has considerable effects on adipocytes in vitro.

The effect of BSA on cytokine release implies that adipocytes are driven into a proinflammatory state when incubated with BSA. This proinflammatory state, in turn, may activate signaling pathways in the adipocytes, leading to various adverse effects. It may be possible that the inhibiting effect of BSA on preadipocyte differentiation, found in the present study, was mediated by the increased release of cytokines. TNF-α, for instance, is well known to have suppressing effects on preadipocyte differentiation (11). On the other hand, cytokines like TNF-α are implicated in the induction of insulin resistance in adipocytes (8). Therefore, the results of this study should be taken into consideration when experiments are performed in adipocytes in the presence of BSA, particularly in relation to diabetes or obesity.

Further investigations are necessary to clarify whether BSA itself is inducing these effects on adipocytes or whether these effects are mediated by other substances. For instance, commercially available BSA could contain small amounts of endotoxin. However, we could exclude that the observed effects of BSA were mediated by endotoxin. Endotoxin levels were...
measured in the incubation media containing various batches of BSA, and these were all <0.5 ng/ml. This concentration is below the threshold concentration of purified LPS (1 ng/ml) required for significant macrophage stimulation (1, 12). Moreover, IL-6 and TNF-α gene expression was stimulated to the same extent with all batches of BSA used in this study, although these batches varied in endotoxin content (data not shown). Thus it is rather unlikely that fatty acids are involved in this process.

On the other hand, BSA in combination with glucose is able to form advanced glycation end products and these glycation products have been demonstrated to be involved in inflammation. However, the conditions in the incubation medium were probably not suitable and the duration of the incubations was not long enough for an appropriate formation of advanced glycation end products (14).

Moreover, there is a possibility that BSA is not regulating the release of cytokines, but is only acting as a stabilizer in the incubation medium and preventing the degradation of the cytokines. Indeed, the effect on cytokine secretion seems to be higher compared with gene expression and, therefore, BSA may also function as a stabilizer. In two experiments (data not shown), the time course of cytokine secretion at a BSA concentration of 2.5 μM for IL-6 and TNF-α was detected. The secretion of IL-6 into the media increased with time, in contrast to mRNA levels. TNF-α values decreased in the medium over time. Therefore, stabilization by BSA seems to be less important for TNF-α than for IL-6. To what extent BSA functions as a stabilizer must be addressed in further studies. This, however, cannot explain the effects of BSA at the level of gene expression of IL-6 and TNF-α. Thus it is rather likely that BSA particularly exerts a direct stimulatory effect on gene expression as has been shown for other cell types (12, 17). The
mechanisms that may underlie this phenomenon are unknown and require elucidation.

In a recent study (5) in humans, an interstitial albumin concentration of 7.36 g/l or 110 μM was measured in adipose tissue. The concentrations that we used in the in vitro experiments fully covered the physiological range. However, it is interesting to note that some of the effects of albumin were already found at concentrations far lower than measured in the above-mentioned study, clearly suggesting that the effects described here could be of physiological significance.

Recent studies (6, 14) have shown that the preparation of fat cells is associated with the induction of proinflammatory pathways leading to an increased release of cytokines such as TNF-α and others. Our data now provide evidence that the frequently used BSA is another condition that may affect cytokine expression and, subsequently, differentiation. However, the latter association is only speculative and requires further examination.

It is interesting to note that the stimulatory effect of BSA on cytokine expression is only transient (Fig. 4). After 48 h, the presence of BSA appears to have only a minor effect on cytokine release from in vitro differentiated human preadipocytes. Thus, depending on the culture conditions, the confounding effect of BSA may be more significant in freshly isolated, mature adipocytes compared with in vitro differentiated preadipocytes.

In conclusion, this study provides evidence that the use of BSA in the incubation medium for the culture of human preadipocytes and adipocytes has substantial consequences for the capacity of adipose differentiation and cytokine production and release. This aspect needs to be adequately considered in future in vitro studies of adipocyte metabolism.

Table 1. Concentration of endotoxin in various incubation media

<table>
<thead>
<tr>
<th>Medium Type</th>
<th>Concentration, ng/ml</th>
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<tbody>
<tr>
<td>Standard adipogenic</td>
<td>0.000</td>
</tr>
<tr>
<td>Standard adipogenic + 0.02% BSA standard grade</td>
<td>0.001</td>
</tr>
<tr>
<td>Standard adipogenic + 0.02% BSA essentially fatty acid free (≥96%)</td>
<td>0.365</td>
</tr>
<tr>
<td>Standard adipogenic + 0.02% BSA highly purified, essentially fatty acid free and essentially globulin free (min. 99%)</td>
<td>0.077</td>
</tr>
<tr>
<td>Standard adipogenic + 0.02% BSA low endotoxin</td>
<td>0.009</td>
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BSA, bovine serum albumin.
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REFERENCES