Haptoglobin release by human adipose tissue in primary culture

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Abstract Haptoglobin is a putative adiposity marker because its concentration in blood is increased in obese humans. The present studies examined haptoglobin release by explants of adipose tissue in primary culture. Haptoglobin was released by explants of human visceral and subcutaneous adipose tissue at a nearly linear rate over 48 h. Explants of visceral adipose tissue released more haptoglobin than did explants of subcutaneous adipose tissue. The release of haptoglobin was quite variable, but there was a close correlation between haptoglobin release by visceral adipose tissue and that by explants of subcutaneous tissue from the same individual. Dexamethasone and niflumic acid, a cyclooxygenase-2 inhibitor, both inhibited haptoglobin release. There was release of haptoglobin by both isolated adipocytes and the adipose tissue matrix remaining after collagenase digestion of human adipose tissue. However, the amount of haptoglobin released by human adipose tissue explants in primary culture was quite low in relationship to the circulating level of haptoglobin.—Fain, J. N., S. W. Bahouth, and A. K. Madan. **Haptoglobin release by human adipose tissue in primary culture. *J. Lipid Res.* 2004.** 45: **536–542.**

Supplementary key words dexamethasone • interleukin-6 • leptin • interleukin-8 • adiponectin • interleukin-1β • niflumic acid • lipopolysaccharide

A proteomics approach to protein secretion by murine 3T3L1 adipocytes in culture suggested that the primary protein released by these cells was haptoglobin (1). A genomic approach resulted in a similar finding, because haptoglobin mRNA was found to be significantly upregulated in the adipose tissue of obese rodents (2, 3). Furthermore, haptoglobin is present at increased levels in the blood of obese humans (4, 5). The circulating levels of haptoglobin in blood (~2 mg/ml in obese humans) are manyfold greater than those of leptin or adiponectin, which are adipokines released by adipose tissue (6–8).

Friedrichs et al. (9) reported that haptoglobin mRNA was present in murine adipose tissue at concentrations 10–15% of that in liver. Treatment of mice with lipopolysaccharide (LPS) at 24 to 30 h before killing increased the haptoglobin mRNA content in adipose tissue to levels comparable to those in liver. Friedrichs et al. (9) also reported that haptoglobin mRNA could be detected in adipocytes but not in capillary or smooth muscle cells of mouse adipose tissue after LPS treatment.

Haptoglobin is an acute phase protein whose synthesis by the liver is increased during inflammation (10). The role of blood haptoglobin is unclear, but one known function is its ability to bind hemoglobin (11). The complex of haptoglobin bound to hemoglobin is then taken up by the liver and is degraded with retention of iron. Free hemoglobin may be toxic under some circumstances because of its ability to degrade nitric oxide and/or because the uptake of hemoglobin by endothelial cells results in the release of free iron that sensitizes cells to damage by oxidants (12, 13). Edwards et al. (14) demonstrated that the factor in plasma responsible for the rapid degradation of nitric oxide was the complex of haptoglobin-hemoglobin. The present studies were designed to determine if haptoglobin is released by human adipose tissue explants in primary culture, whether more haptoglobin is released by visceral than by subcutaneous adipose tissue, and what regulates haptoglobin release by adipose tissue.

MATERIALS AND METHODS

Abdominal subcutaneous and visceral adipose tissues were obtained from women with an average body mass index (BMI) of 33 who were undergoing open abdominal surgery (abdominoplasty) or patients who were undergoing laparoscopic gastric bypass with Roux-en-Y gastroenterostomy surgery for the treatment of morbid obesity with an average BMI of 45. Each experimental replication involved tissue from a separate individual. The study had the approval of the local institutional review board, and all patients involved gave their informed consent. The patients were on a clear liquid diet the day before surgery but had not been on any type of dietary restriction before surgery.

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Ten to 20 g of visceral and subcutaneous adipose tissue were immediately transported to the laboratory. The handling of tissue and cells was done under aseptic conditions. The tissue was cut with scissors into small pieces (20–30 mg). All of the studies used explants of adipose tissue that had been incubated in buffer plus albumin (3 ml/g tissue) for ~30 min to remove diffusible factors and blood cells. At the conclusion of the 30 min incubation, the tissue explants were centrifuged for 30 s at 400 *g* to remove erythrocytes and pieces of tissue containing insufficient adipocytes to float. The explants were separated from the medium, and the sedimented cells and tissue fragments were then resuspended in fresh buffer. The explants (500 mg in 5 ml) were incubated in duplicate for 4, 24, or 48 h in suspension culture under aseptic conditions.

To obtain isolated adipocytes, 1 g of cut tissue, in duplicate, was incubated in 2 ml of incubation medium containing 1.3 mg of bacterial collagenase in a rotary water bath shaker (100 rpm) for 2 h. The collagenase digest was then separated from undigested tissue matrix by filtration through 200 μm nylon mesh fabric. Five milliliters of medium was then added back to the digestion tubes and used to wash the undigested matrix on the nylon mesh. This wash solution was combined with the collagenase digest, and stromal-vascular (SV) cells were separated from adipocytes and medium by centrifugation in 15 ml tubes for 1 min at 400 *g*. The SV cells and adipocytes were separately resuspended in 5 ml of fresh buffer and centrifuged for 10 s at 400 *g*. The medium was removed, and the undigested tissue matrix on the nylon mesh, the SV cells, and the adipocytes were then incubated in a volume of 5 ml for the indicated periods.

The buffer for incubation of adipose tissue and adipocytes was as previously described (15). Aliquots of the medium were taken at 48 h and stored at −20°C for measurement of release to the medium. The pooled tissue explants (1 g) were homogenized for analysis of haptoglobin using a Polytron (15 s at setting 9) in 2 ml of incubation buffer plus 4 μl of a protease inhibitor cocktail [P1860 from Sigma Aldrich Chemical Co. (St. Louis, MO) containing aprotinin, bestatin, E-64, leupeptin, and pepstatin].

Tumor necrosis factor-α (TNF-α), interleukin-8 (IL-8), and IL-1β were measured using ELISA kits from the Central Laboratory of the Netherlands Red Cross that are distributed by Research Diagnostics (Flanders, NJ). Vascular endothelial growth factor (VEGF) was measured using an ELISA kit from Pierce Biotechnology (Rockford, IL). Leptin and adiponectin were determined using ELISA assays with antibodies and standards obtained from R&D Systems, Inc. (Minneapolis, MN). The capture antibody for the human leptin assay was mouse anti-human antibody, and the detection antibody was biotinylated mouse anti-human antibody (DuoSet kit). The detection antibody for the human adiponectin assay was the IgG fraction of a monoclonal murine antibody purified by protein G affinity chromatography. The capture antibody for the human adiponectin assay was the same antibody that was biotinylated. Prostaglandin E_2 (PGE_2) was assayed as previously described (15).

Haptoglobin was assayed using an ELISA procedure developed in our laboratory. The capture antibody was monoclonal murine anti-human haptoglobin antibody (clone HG-36 from Sigma-Aldrich used as a 1:1,000 dilution of mouse ascites fluid). The detection antibody was the biotinylated purified IgG fraction of a polyclonal sheep anti-human haptoglobin obtained from Accurate Chemical and Scientific Corp. (Westbury, NY). The haptoglobin assay could detect 20 pg in 100 μl of medium. The standard for the haptoglobin assay was human plasma haptoglobin of mixed type obtained from Calbiochem Corp. (La Jolla, CA) or ICN Biomedicals, Inc. (Aurora, CA), which was dissolved in incubation buffer containing 1% albumin. Aliquots of the haptoglobin standard were stored at −80°C and used only once. We attempted to use a haptoglobin preparation from another supplier prepared by dissociation of the hemoglobin-haptoglobin complex using high concentrations of urea or guanidine (16), but the immunoreactivity was 10% of that using haptoglobin obtained by Cohn fractionation of plasma. The ELISA plates were high-binding polystyrene 96-well flat-bottom microtiter plates (Immulon 2HB; Thermo Systems, Franklin, MA). The blocking buffer was SuperBlock™ from Pierce Biotechnology, as were the streptavidin conjugated to horseradish peroxidase and the TMB substrate for color development.

All values shown are means ± SEM. Pearson correlation coefficients were determined using the GraphPad Prism program assuming a Gaussian population and a two-tailed *P* value.

RESULTS

Haptoglobin circulates in blood at concentrations in the range of milligrams per milliliter. However, the release of immunoreactive haptoglobin to the medium by human adipose tissue over 48 h was 40 ng/g adipose tissue in the studies shown in **Fig. 1A** and 120 ng/g in Fig. 1B. The explants of adipose tissue had first been preincubated for 30 min in buffer to reduce the amount of haptoglobin present in the cut tissue. The data shown in Fig. 1A demonstrate that this was successful, as less than 10% of the haptoglobin that accumulated in the medium over 48 h was initially present in tissue explants. The release of hap-

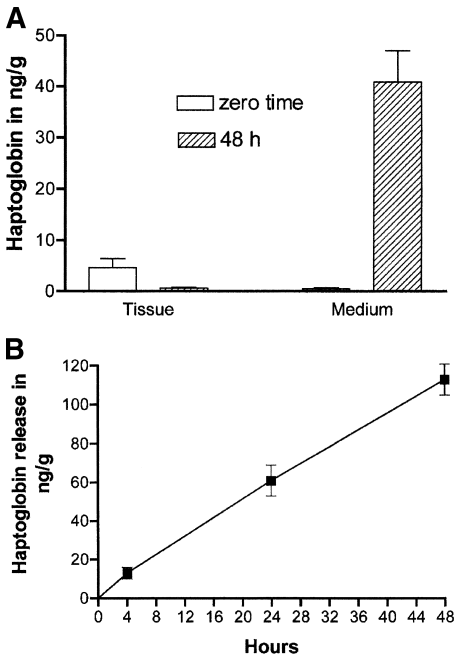

Fig. 1. Haptoglobin release to the medium by human adipose tissue explants in primary culture. A: Tissue explants (100 mg/ml) were incubated for 48 h, and the values shown are means ± SEM of 12 paired experiments (6 using subcutaneous and 6 using visceral adipose tissue). The average body mass index (BMI) of the tissue donors was 45. B: Explants of human adipose tissue (100 mg/ml) were incubated for 4, 24, or 48 h, and the values shown are means ± SEM of 24 paired experiments (12 using subcutaneous and 12 using visceral adipose tissue). The average BMI of the tissue donors was 41.

toglobin by tissue explants was fairly linear over a 48 h incubation (Fig. 1B).

The release of haptoglobin by explants of subcutaneous adipose tissue varied enormously from one individual to the next (**Fig. 2A**). However, there was a highly significant correlation between haptoglobin release by explants of visceral adipose tissue over 48 h and that by explants of subcutaneous adipose tissue from the same individual (Pearson correlation coefficient of 0.89, $P < 0.001$).

The release of haptoglobin per gram of tissue by visceral adipose tissue explants was greater that that by subcutaneous explants based on paired comparisons in both the abdominoplasty patients with an average BMI of 33 and the gastric bypass patients with an average BMI of 45 (Fig. 2B). As a result of the wide variability in release from one individual to the next, there was no statistically significant difference in release per gram of tissue between the two groups of patients (Fig. 2B).

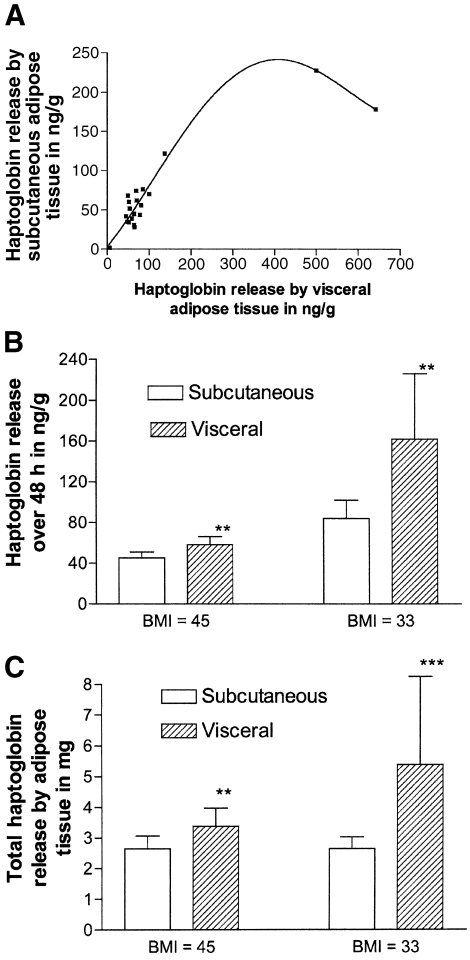

Fig. 2. Visceral adipose tissue releases more haptoglobin than does subcutaneous adipose tissue. A: The release by explants of visceral adipose tissue was compared with that by subcutaneous adipose tissue from the same donors over 48 h for 22 individuals with BMI values ranging from 27 to 50. The Pearson correlation coefficient was 0.89, and $P < 0.001$. The line connecting the points was based on polynomial second-order curve fitting ($r^2 = 0.93$). B and C: Data are shown as means ± SEM for 12 paired samples from gastroplasty donors with an average BMI of 45 and for 10 paired samples from abdominoplasty donors with an average BMI of 33. In B, the data are shown as nanograms per gram, and in C, the data are shown as total release corrected for the amount of total body fat. Statistically significant differences between subcutaneous and visceral fat samples based on paired comparisons are indicated by asterisks (** $P < 0.025$ and *** $P < 0.005$).

The difference between haptoglobin release by visceral versus subcutaneous adipose tissue expressed per gram of adipose tissue was apparently not attributable to visceral adipose tissue having more adipocytes per gram of tissue. In six paired experiments, the average adipocyte cell yield from visceral adipose tissue was 57% compared with 67% in subcutaneous adipose tissue, suggesting that adipocytes constitute a smaller fraction of visceral adipose tissue. The average cell diameter of subcutaneous adipocytes obtained by collagenase digestion from nine gastric bypass patients with a mean BMI of 45 was 121 μm, which means that there were 1×10^6 cells per gram wet weight. In contrast, the diameter of visceral adipocytes from the same individuals was 108 μm, which was 11% less than that of subcutaneous adipocytes. The diameter of subcutaneous adipocytes from six abdominoplasty patients was 102 μm, whereas that of visceral adipocytes was 88 μm, which was 14% less that that for subcutaneous adipocytes. The abdominoplasty patients in this series had a mean body fat content of 33 kg, whereas the gastric bypass patients had 58 kg. If we then multiplied haptoglobin release per gram by the total amount of fat, we obtained the values shown in Fig. 2C, but again, there was no statistically significant difference in haptoglobin release between the two groups.

It has been postulated that the enhanced accumulation of haptoglobin mRNA in the adipose tissue of obese rodents is linked to the TNF-α whose release is stimulated by LPS (2). The data shown in **Fig. 3** demonstrate that the direct addition of LPS from *Escherichia coli* to explants of human adipose tissue did not enhance haptoglobin, leptin, or adiponectin release under conditions in which it did enhance IL-6, IL-8, and IL-1β release. We also obtained negative effects on haptoglobin release after the direct addition of TNF-α at a concentration of 10 ng/ml to explants of human adipose tissue, because the percentage change attributable to the TNF-α after 48 h was 0 ± 15% (mean ± SEM of six paired experimental replications).

Dexamethasone, which inhibits the upregulation of cyclooxygenase-2 in human adipose tissue (15), inhibited the release of haptoglobin as well as the release of IL-6, IL-8, IL-10, IL-1β, VEGF, and PGE_2 under conditions in which it enhanced the release of leptin and had no effect on adiponectin release (Fig. 3). Niflumic acid is a potent cyclooxygenase-2 inhibitor with a K_i for the human recombinant enzyme of 100 nM (17). Niflumic acid at a concentration of 100 nM reduced the release of haptoglobin as well as the release of PGE_2, IL-6, IL-8, IL-10, leptin, IL-1β, and VEGF (Fig. 3). Niflumic acid did not affect the release of adiponectin (Fig. 3).

The basal values for the release of adipokines and other factors by human subcutaneous adipose tissue explants over 48 h from the experiments shown in Fig. 3 are shown in **Fig. 4**. These data indicate that the release of haptoglobin was ~1% of IL-8, 5% of IL-6, 8% of adiponectin, and

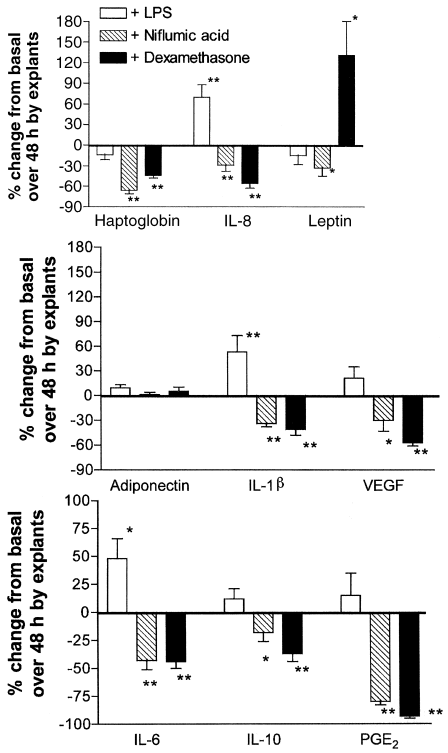

Fig. 3. Comparison of the effects of dexamethasone, niflumic acid, and lipopolysaccharide (LPS) on haptoglobin, adiponectin, interleukin-6 (IL-6), IL-8, IL-10, IL-1β, vascular endothelial growth factor (VEGF), leptin, and prostaglandin E_2 (PGE_2) release by explants of human adipose tissue. Explants of human subcutaneous adipose tissue (100 mg/ml) were incubated for 48 h in the presence of 25 μg/ml LPS from *Escherichia coli* (055:B5), niflumic acid at 100 nM, or dexamethasone at 10 nM. The values shown are means ± SEM of the percentage changes from basal in eight paired experiments. The basal values in the absence of added agents are shown in Fig. 4. Statistically significant effects of added agents are indicated as follows based on paired comparisons: * $P < 0.05$ and ** $P < 0.025$.

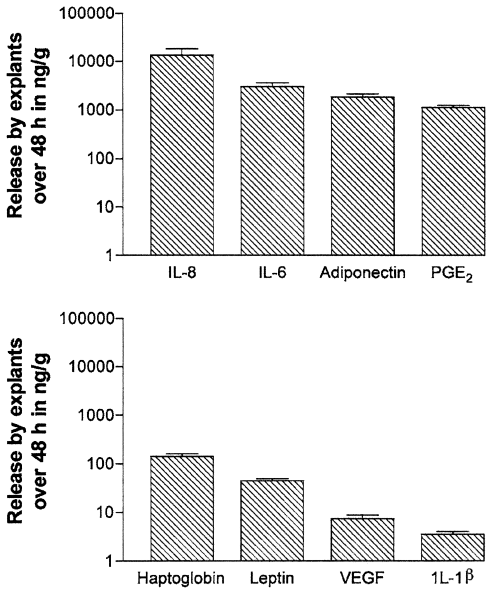

Fig. 4. Haptoglobin release by human adipose tissue explants compared with that of IL-8, IL-6, IL-1β, VEGF, leptin, PGE_2, and adiponectin. Explants of human subcutaneous adipose tissue (100 mg/ml) were incubated for 48 h. The values shown are means ± SEM of eight paired experiments, and the average BMI of the tissue donors was 30.

13% of PGE_2 release. In contrast, more haptoglobin was released than was the case for leptin, VEGF, or IL-1β (Fig. 4).

Resistin is primarily released by the nonfat cells of human adipose tissue (18). We wanted to determine if this was also the case for haptoglobin and compared its release with that of leptin, IL-6, and TNF-α by the adipose tissue matrix, the isolated SV cells, and the adipocytes obtained from 1 g of adipose tissue (**Fig. 5**). The adipose tissue matrix is the washed cellular debris remaining after collagenase digestion of adipose tissue explants that does not pass through a 200 μm mesh filter. The SV cells are those cells that pass through the filter and are sedimented by brief centrifugation, whereas the adipocytes are those cells that pass though the filter but float after centrifugation. Over a 4 h incubation, haptoglobin release by the cells of the adipose tissue matrix was 66% greater than that by adipocytes, and at 24 h, the release of haptoglobin was 52% greater than that by adipocytes. There was virtually no release of haptoglobin by SV cells. In contrast, TNF-α release was greatest by the SV cells, and there was little release by adipocytes (Fig. 5). With IL-6, a different pattern of release was seen, with most of the IL-6 coming from the cells present in the undigested tissue matrix. However, leptin release by the adipocytes was much greater than that by adipose tissue matrix or SV cells (Fig. 5). These data indicate that leptin is primarily made by mature adipocytes that are released by collagenase digestion, TNF-α by SV cells, and IL-6 by cells in the adipose tissue matrix, whereas haptoglobin is released by both adipocytes and the cells of the adipose tissue matrix.

DISCUSSION

Scriba et al. (4) reported that the concentration of haptoglobin in the blood of obese humans was 2.5 ± 1.5 mg/ml. Control values in nonobese individuals ranged from 0.25 to 2.5 mg/ml. Hannerz, Greitz, and Ericson (5) subsequently confirmed this finding. Haptoglobin is an acute phase protein primarily made by the liver in response to inflammation. It is thought that IL-6 is the major cytokine that stimulates the synthesis in liver of acute phase proteins, rather that TNF-α (10, 19). A plausible explanation for the increase of circulating haptoglobin in obesity is the finding that plasma levels of IL-6 are increased in obesity (20, 21).

The present results demonstrate that haptoglobin is released by human adipose tissue explants in primary culture, but the amount released is quite small in relationship to circulating values. The calculated total release of haptoglobin over 48 h by adipose tissue explants in mark-

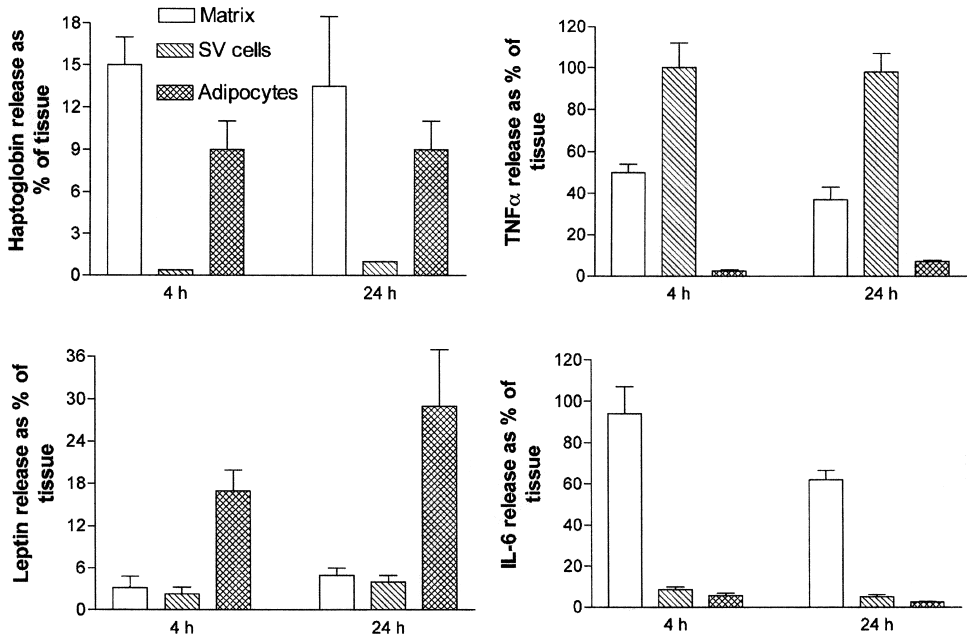

Fig. 5. Comparison of haptoglobin, leptin, tumor necrosis factor-α (TNF-α), and IL-6 release by adipose tissue matrix, stromal-vascular (SV) cells, and adipocytes compared with that by tissue explants. Explants of human subcutaneous tissue (100 mg/ml) as well as the undigested adipose tissue matrix, SV cells, and adipocytes obtained after collagenase digestion were incubated for 4 or 24 h. The data shown are means ± SEM of four paired experiments, and the BMI of the tissue donors was 44. The release by adipocytes, SV cells, and the adipose tissue matrix is expressed as a percentage of that by the same amount of adipose tissue not treated with collagenase. The data are uncorrected for the loss of cells during collagenase digestion or during the separation and washing of the fractions. The total recovery of lactate formation expressed as a percentage of that by tissue explants at 24 h was 46% for the tissue matrix fraction, 9% for the SV cells, and 9% for the adipocytes. The release of haptoglobin by tissue explants was 16 ng/g at 4 h and 37 ng/g over 24 h. Leptin release at 4 h was 8 ng/g, whereas at 24 h it was 16 ng/g. TNF-α release at 4 h was 3.5 ng/g, and at 24 h it was 3.1 ng/g. IL-6 release was 500 ng/g at 4 h and 4,300 ng/g at 24 h.

edly obese individuals with a body fat content of 58 kg was approximately equivalent to the amount present in 1 ml of plasma. Furthermore, the ratio of release over 48 h by 1 g of adipose tissue (120 ng/g) divided by the serum value for haptoglobin in obese humans of 2,200,000 ng/ml (4) was 5×10^{-5}. In contrast, the release of leptin was 45 ng/g over 48 h and the serum value for leptin in obese individuals was 74 ng/ml (22), giving a ratio of 0.6. These data suggest that the contribution of adipose tissue to circulating haptoglobin values is minor in contrast to leptin, which is primarily a product of adipose tissue. However, the release of haptoglobin by adipose tissue explants may be less than that seen in vivo by adipose tissue, and the half-life of plasma haptoglobin may be far greater than that of leptin. Future studies should compare the release of haptoglobin by human liver cells in vitro with that by adipose tissue.

The finding that direct addition of LPS to human adipose tissue explants did not stimulate haptoglobin release under conditions in which it stimulated that of IL-8, IL-6, and IL-1β is of interest. There is so much release to the medium by human adipose tissue explants of immunoreactive IL-6 and IL-8 that any further increase attributable to LPS may have little effect on haptoglobin formation. With IL-1β, the situation is different, because the basal release resulted in a final concentration of only 0.36 ng/ml after 48 h, which was increased to 0.54 ng/ml in the presence of LPS. The addition of 1 ng/ml IL-1β at the start of a 48 h incubation increased haptoglobin release by more than 300% (our unpublished observations). The LPS receptor is the Toll-like receptor 4 that is present not only in macrophages but also in murine adipose tissue, 3T3 cells, and 3T3L1 adipocytes (23). The LPS data suggest that the marked increase in adipose tissue haptoglobin mRNA content seen after treatment of intact mice with LPS (9) was secondary to the enhanced release of inflammatory mediators such as IL-1β, IL-6, TNF-α, or other cytokines by the nonfat cells of adipose tissue. Species differences may also be involved, because haptoglobin appears to be the major protein released by 3T3L1 adipocytes (1), whereas in human adipose tissue, it is released in amounts far less than those of IL-8, IL-6, or adiponectin.

Dexamethasone inhibited the release of haptoglobin, IL-8, IL-6, IL-1β, IL-10, VEGF, and PGE_2 but had no effect on adiponectin release and stimulated leptin release. Zhang et al. (24) reported that dexamethasone inhibits IL-1β mRNA accumulation by human adipocytes, and our results extend this finding to IL-1β release. The stimulation of leptin release and the inhibition of PGE_2 are in agreement with previous studies in which it was also shown that dexamethasone blocked the upregulation of cyclooxygenase-2 seen during a 48 h incubation of human

adipose tissue explants (15). The inhibition of haptoglobin release by niflumic acid, which is a cyclooxygenase-2 inhibitor (17), suggests that haptoglobin release, like that of the cytokines released by adipose tissue, requires cyclooxygenase-2 activity. However, it is possible that the inhibition of haptoglobin release by the cyclooxygenase inhibitor is attributable to the reduced formation of reactive oxygen species generated by cyclooxygenase-2, as suggested by Viviani et al. (25).

The traditional view that haptoglobin is an acute phase protein made by the liver must now be modified by our data showing its expression in adipose tissue. This confirms reports that haptoglobin is also made by intestinal epithelial cells (26), reactive astrocytes after transient forebrain ischemia in rats (27), as well as cancers (28). The demonstration that haptoglobin is made by nonhepatic cells does not contradict the paradigm that circulating haptoglobin is derived primarily from the liver, but inflammatory stress may upregulate its local formation in a wide variety of cells. Because the blood level is so high in relationship to the amount secreted by human adipose tissue, it is possible that, as in neutrophils, haptoglobin is taken up by cells in adipose tissue and subsequently secreted during short-term incubations (29). However, over a 48 h incubation of human adipose tissue explants, we found that more than 90% of the haptoglobin released was derived from de novo synthesis.

On the basis of studies using murine 3T3 cells, it was suggested that haptoglobin is primarily made by differentiated adipocytes rather than preadipocytes (1). Furthermore, the level of haptoglobin mRNA is increased in obese mice, and this has been attributed to increased levels of TNF-α (2). Chiellini et al. (2) also reported that immunohistochemical analysis indicated positive cytoplasmic staining for haptoglobin mRNA in adipocytes but not in endothelial cells of the capillaries or other cell types of both human and murine adipose tissue. Our data indicate a release of haptoglobin by human adipocytes as well as a substantial release of haptoglobin, but not of leptin, by the cells present in the matrix remaining after collagenase digestion of human adipose tissue. In view of the low recovery of leptin and haptoglobin synthesis by isolated adipocytes and matrix over 4 or 24 h, it is possible that their formation is either downregulated or damaged by collagenase digestion. However, we did not correct for cell loss during collagenase digestion, and lactate formation by the matrix, SV cells, and adipocytes was 47, 9, and 9%, respectively, of that by adipose tissue, indicating a recovery of ~65% of lactate formation per gram of adipose tissue. This was similar to what we saw with respect to IL-6 release. TNF-α formation was upregulated, because formation by the three fractions over 24 h was greater than that by tissue explants.

Our finding that haptoglobin is made by the nonfat cells of human adipose tissue is similar to what we saw with resistin (18). We conclude that haptoglobin is released in small amounts by human adipose tissue explants but, as with resistin, more is made by the nonfat cells in human adipose tissue than is made by adipocytes.

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