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Involvement of multiple signaling pathways in the post-bariatric induction of IL-6 and IL-8 mRNA and release in human visceral adipose tissue

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Abstract

The present studies were designed to determine the site of and the mechanism for the rapid increase in IL-6 and IL-8 mRNA observed in human visceral adipose tissue after removal during laparoscopic bariatric surgery. Upregulation of IL-6 and IL-8 mRNA as well as their release were seen within 3 h whether one intact piece of tissue or minced pieces of adipose tissue were incubated in vitro. Most of the IL-6 and IL-8 mRNA content of visceral adipose tissue after 3 h of incubation was in the non-fat cells. Actinomcyin D markedly reduced the upregulation of IL-6 and IL-8 mRNA. Incubation of adipose tissue explants with a soluble TNFα receptor (etanercept) plus a blocking antibody against IL-lβ reduced by 55% the increase in IL-6 mRNA and by 42% that of IL-8 mRNA seen between 1 and 5 h of incubation. The upregulation of IL-8 and IL-6 mRNA accumulation as well as their release over a 2 or 4 h incubation was reduced by around 50% in the presence of an inhibitor of the p38 MAPK or an inhibitor of the NFκB pathway and by 85% in the presence of both inhibitors. The data suggest that the relative trauma and/or hypoxia that occurs when adipose tissue is removed results in the release of TNFα and IL-1β. These cytokines, and probably other factors as well, enhance IL-6 and IL-8 mRNA accumulation in human adipose tissue explants through mechanisms involving the p38 MAPK and NFκB pathways
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Keywords: IL-6; IL-8; NFKB; TNFα; IL-1β; p38 MAPK

1. Introduction

Circulating levels of IL-6 are elevated in obesity and reduced after weight loss [1–3]. Recently, Ruan et al. [4] reported that after removal of adipose tissue from mice there was a marked upregulation of IL-6 mRNA in adipocytes. Other mediators of IL-6 gene expression in adipose tissue of humans are a meal [5] or insulin administration [6,7].

IL-8 is another cytokine/chemokine whose circulating level is elevated in obese humans [2,8]. No studies have been have been published on the regulation in murine adipose tissue of macrophage inflammatory protein 2 which is the rodent homologue of IL-8 and secreted by murine macrophages in response to bacterial lipopolysaccharide [9]. More IL-8 is released by adipose tissue explants or adipocytes over a 4 h incubation than any other adipokine [10]. IL-8 release by explants of human adipose tissue correlated with the body mass index of the donors from which the tissue was obtained and this enhanced release was not seen in adipocytes [11]. There is also more release of IL-8 by visceral than by explants of human subcutaneous adipose tissue [11]. The enhanced release of IL-8 could be involved in the recruitment of monocytes to adipose tissue where they are transformed into macrophages.

Trayhurn and Wood [12] have recently suggested that the mild inflammatory state seen in obesity that is characterized by elevated circulating levels of IL-6 is a response to hypoxia. Adipose tissue blood flow is markedly reduced in obese humans [13,14] and obesity decreases perioperative tissue oxygenation [15]. In human astrocytes,

Abbreviations: SV, stromovascular; TNFα, tumor necrosis factor alpha; NFκB, nuclear factor κB; p38 MAPK, p38 mitogen-activated protein kinase; JNK, Jun N-terminal kinase; ERK, p44/42 mitogen-activated protein kinase; TGF-β1, transforming growth factor-β1

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it has been shown that hypoxia upregulates IL-8 by a mechanism involving release of IL-1β [16]. In human ovarian carcinoma cells, hypoxia enhances IL-8 expression via p38 MAPK [17]. An induction by hypoxia of IL-6 in cardiac myocytes has also been reported and attributed to NFκB activation [18]. These reports prompted our interest in determining whether the release of IL-6 and IL-8 is upregulated ex vivo after removal of human visceral adipose tissue, whether IL-1β is involved in upregulation of these cytokines, whether the p38 MAPK and NFKB pathways are involved in regulation of IL-6 and IL-8 release by human adipose tissue and the role of hypoxia.

For the present studies, we utilized both intact (minced as well as one intact piece) and collagenase-prepared fractions of visceral adipose tissue obtained from morbidly obese women undergoing laparoscopic bariatric surgery. Our measurements were taken at the earliest possible time points in order to minimize the effect of removal of adipose tissue from humans and the effects of collagenase digestion on the various adipose tissue fractions.

2. Materials and methods

Visceral adipose tissue was obtained from obese women undergoing laparoscopic adjustable gastric banding surgery or laparoscopic gastric bypass with Roux-en-y gastroenterostomy surgery for the treatment of morbid obesity. The average body mass index of the fat donors for each set of experiments is shown in the figure or table legends. Approximately 73% of the patients were age 40 years or less, 12% were ages 40–49 years and 15% were over the age of 50 years and the average age was 38. Each experimental replication involved tissue from a separate individual. Fasting blood glucose values exceeded 125 mg/dl in approximately 10% of the patients. The study had the approval of the local IRB and all patients involved gave their informed consent. The patients were on a liquid diet for two weeks prior to surgery.

The visceral adipose tissue was transported to the laboratory within 15–30 min of its removal from the donor. The handling of tissue and cells was done under aseptic conditions. The tissue was cut with scissors into small pieces (5–10 mg). All the studies utilized explants of adipose tissue that had been incubated in buffer plus albumin (3 ml/g of tissue) for approximately 5–30 min to reduce contamination of the tissue with blood cells and soluble factors. At the conclusion of this incubation, the tissue explants were centrifuged for 30 s at $400 \times g$ in order to remove blood cells and pieces of tissue containing insufficient adipocytes to float. The explants (100 mg/ml) were then incubated in duplicate for the indicated times in suspension culture under aseptic conditions.

Adipocytes were prepared by incubating 0.5 g of cut adipose tissue per ml of incubation medium containing 0.6 mg/ml 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 mesh fabric. Five ml of medium was then added back to the digestion tubes and used to wash the undigested matrix on the filter mesh. This wash solution was combined with the collagenase digest and SV cells were separated from adipocytes and medium by centrifugation in 15 ml tubes for 1 min at $400 \times g$. The SV cells are defined as those cells isolated by collagenase digestion that do not float. The SV cells and adipocytes were each suspended in 5 ml of fresh buffer and centrifuged for 10 s at $400 \times g$. 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 serum-free buffer for incubation of adipose tissue and adipocytes was as previously described [10,19]. The pH of the buffer was adjusted to 7.4 and then filtered through a 0.2 μm filter. IL-6 and IL-8 were determined using ELISA assays with Duoset reagents from R&D Systems of Minneapolis, MN.

For studies involving mRNA isolation, the matrix, cells or tissue were separated from the medium and RNA extracted by Polytron homogenization using 5 ml of a monophasic solution of phenol and guanidine isothiocyanate (TRIzol reagent from Invitrogen, Carlsbad, CA). The extracts were then spun at $12{,}000 \times g$ for 10 min at 2–8 °C to separate the fat from the extract. Total RNA was obtained from the cleared homogenate by the procedure of Chomczynski and Sacchi [20]. The amount of extracted RNA was determined by absorption at 260 nm. The total recovery of RNA in the undigested tissue matrix was 25% of that obtained from one gram of visceral adipose tissue, while that in adipocytes was 20%. The quantitation of mRNA was based on hybridization of samples using gene-specific biotin-labeled capture oligonucleotide probes and digoxigenin-labeled detection probes in a microplate. The hydribidization solution was then transferred to a strepavidin-coated microplate and the amount of the digoxigen labeled probe bound to the strepavidin plate detected by use of an anti-digoxigen alkaline phosphatase complex (Quantikine mRNA kits from R&D Systems, Minneapolis, MN). The sensitivity of this procedure is comparable to that of Northern blots.

We used 6-amino-4-phenoxyphenethylaminoquinazoline as an inhibitor of NFκB transcriptional activation [21]. The IC_{50} for this effect in Jurkat cells is 11 nM, while the IC_{50} for cellular toxicity is around 10 μM according to the manufacturer. The JNK inhibitor II, also known as SP 600125, is an anthrapyrazolone and inhibits JNK 1, 2 or 3 kinases with an IC_{50} of less than 0.1 μM, while the IC_{50} for inhibition of other kinases, such as p38 MAPK or ERK, is greater than 10 μM [22]. UO126 is another small molecular weight organic compound that inhibits MEK-1 and MEK-2 kinases that activate ERK [23]. This compound inhibits the MEK kinases with an

IC_{50} of around 60 nM and ERK phosphorylation in COS-7 cells with an IC_{50} of around 0.1 μM; while for other kinases, the IC_{50} is above 10 μM [24]. SB 220025 is a pyrimidinyl imidazole compound that inhibits p38 MAPK with an IC_{50} of 60 nM and 50–1000-fold selectivity versus other protein kinases [25]. We used the drugs at a concentration of 1 μM. We also examined the p38 MAPK and NFκB inhibitors at a concentration of 0.2 μM, where their effects over a 4 h incubation of visceral adipose tissue were 62–73% of those seen at a concentration of 1 μM on either IL-6 or IL-8 release (unpublished data, Fain, Bahouth and Madan).

Bovine serum albumin powder (Bovuminar, containing <0.05 mol of fatty acid/mole of albumin) was obtained from Intergen (Purchase, NY). Bacterial collagenase *Clostridium histolyticum* (Type 1) was obtained from Worthington Biochemical Corporation (lot CLS1-4197-MOB3773-B, 219 U/mg). Etanercept (Enbrel®) was manufactured by Immunex Corp., Seattle, WA. The monoclonal anti-human IL-1β was purified using Protein A affinity chromatography and obtained from a mouse hybridoma (clone 8516.311). It was obtained from Sigma Chemical Co., St. Louis, MO. The ERK inhibitor, UO-126 [1,4-diamino-2,3-dicyano-1,4-bis(2-aminophenylthio)butadiene]; the JNK inhibitor II {anthra[1,9-*cd*]pyrazol-6(2*H*)-one; 1,9-pyrazoloanthrone} also known as SP600125, the p38 MAPK inhibitor SB220025 [5-(2-amino-4-pyrimidinyl)-4-(4-fluorophenyl)-1-(4-piperidinyl)imidazole], and the NFκB activation inhibitor 6-amino-4-(4-phenoxyphenethylamino) quinazoline were obtained from EMD/Calbiochem, La Jolla, CA.

3. Results

3.1. Are IL-6 and IL-8 upregulated after removal of adipose tissue from humans?

The possibility that IL-6 and IL-8 mRNA as well as their release were upregulated in adipose tissue was examined in the studies shown in Fig. 1. The IL-6 and IL-8 mRNA content of cut pieces of adipose tissue was approximately 10 amol of RNA per microgram of total RNA in adipose tissue immediately after removal from the fat donors. There were small increases in IL-8 mRNA at 1 h and the subsequent increases in IL-8 mRNA were much greater than those of IL-6 (Fig. 1a). However, the IL-6 mRNA content was not changed when the adipose tissue was minced, then incubated for 30 min and examined at 1 h (Fig. 1b). But by 3 h after removal and mincing of the fat, there was a five-fold increase in the amount of IL-6 mRNA in adipose tissue and this was unchanged at 25 or 49 h. Similar data were seen with regard to IL-8 and IL-6 release by adipose tissue at 3, 24 and 48 h (Fig. 1c and d).

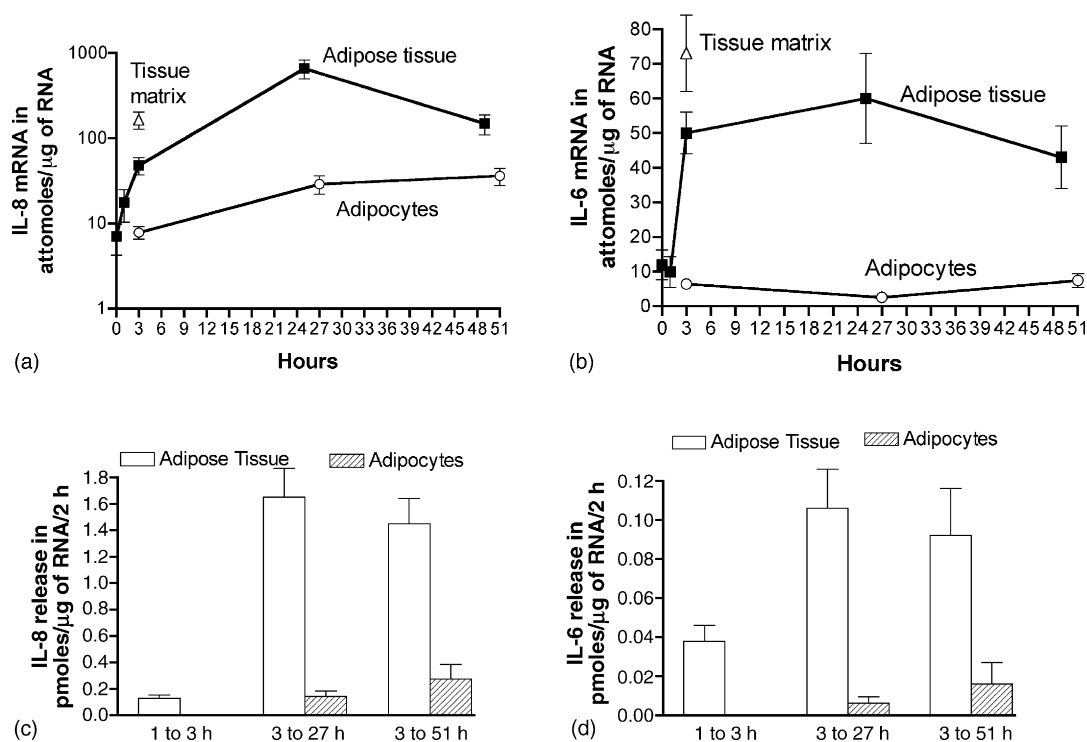

Fig. 1. Upregulation of IL-6 and IL-8 mRNA and release in cut pieces of human adipose tissue. The data are shown as the mean ± S.E.M. for six paired experiments using visceral adipose tissue from six donors with a mean body mass index of 45. The data for mRNA (panels a and b) represent values for tissues taken immediately after removal, 1, 3, 25 or 49 h and for adipocytes at 3, 27 and 51 h. The data for IL-6 and IL-8 release are shown in panels c and d and are for explants incubated for 2, 24 or 48 h starting 1 h after removal from the donors. Adipocytes were incubated for 24 and 48 h starting 3 h after removal from the donors.

We also measured the IL-6 and IL-8 mRNA content of isolated adipocytes at 3, 27 and 51 h after removal of adipose tissue (Fig. 1a and b). The IL-6 mRNA content of adipocytes per microgram of total RNA was 13% of that for tissue at 3 h and 9% of that for the undigested adipose tissue matrix. For IL-8 mRNA, the comparable percentages were 16 and 5%, respectively. These data clearly indicate that most of the upregulation of IL-6 and IL-8 mRNA involves the non-fat cells of human adipose tissue. There was a much greater effect of collagenase digestion on the IL-8 mRNA content of the tissue matrix as compared to the tissue which was measured at 3 h (3.4-fold). This was in contrast to the situation with IL-6 mRNA, where the increase was only 1.5-fold greater in debri than in tissue at 3 h (Fig. 1b). IL-8 mRNA content of adipocytes was increased by 3.7-fold between 3 and 27 h of incubation, in contrast to IL-6 mRNA, which did not increase between 3 and 27 h of incubation (Fig. 1a).

3.2. Does mincing of adipose tissue cause the up-regulation of IL-6 and IL-8 release seen in visceral adipose tissue explants?

The studies shown in Fig. 1 indicated that while collagenase digestion further enhanced the up regulation of IL-6 and IL-8 mRNA in the tissue matrix there was a massive upregulation of IL-8 and to a lesser extent IL-6 mRNA in cut pieces of adipose tissue. We, therefore, examined whether cutting of adipose tissue was responsible for the upregulation shown in Fig. 2. In these studies, the release of IL-6 or IL-8 as well as their mRNA content was examined over 5 h of incubation of either one piece of adipose tissue or minced pieces of adipose tissue. Surprisingly, the release of both IL-6 and IL-8 as well as the accumulation of their mRNAs were not affected by mincing the tissue. All our studies are done using air as the gas phase and minced pieces are ordinarily used to reduce variability as well as to increase the diffusion of oxygen and nutrients to the center of the adipose tissue pieces.

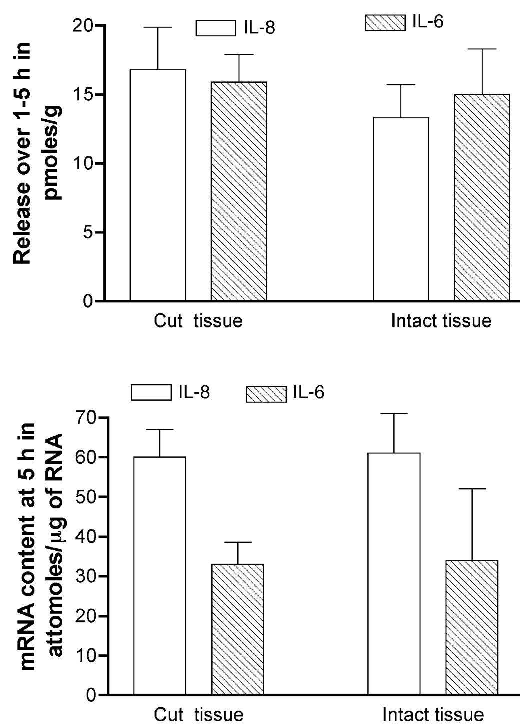

Fig. 2. Upregulation of IL-8 and IL-6 mRNA does not require mincing of visceral adipose tissue. Minced adipose tissue (200 mg in 2 ml of medium) was incubated for 30 min, subjected to brief centrifugation, suspended in fresh medium and then incubated for 4 h starting approximately 1 h after the start of the incubation with intact tissue. One cut piece of adipose tissue (200 mg in 2 ml of medium) was incubated for 5 h and samples of medium were removed at 1 and 5 h to analyze for IL-8 and IL-6 release. The values for release by intact tissue and explants are for the 1–5 h period and mRNA content was determined at the end of the incubation. IL-6 and IL-8 release are the mean ± S.E.M. of 12 experiments and mRNA was analyzed in 8 of the experiments. The 1 h value for mRNA was also analyzed in cut pieces of adipose tissue from these experiments and for IL-6 was 2.8 amol/μg of total RNA and for IL-8 mRNA was 9 amol/μg of RNA. The average body mass index of the fat donors was 52 in these experiments.

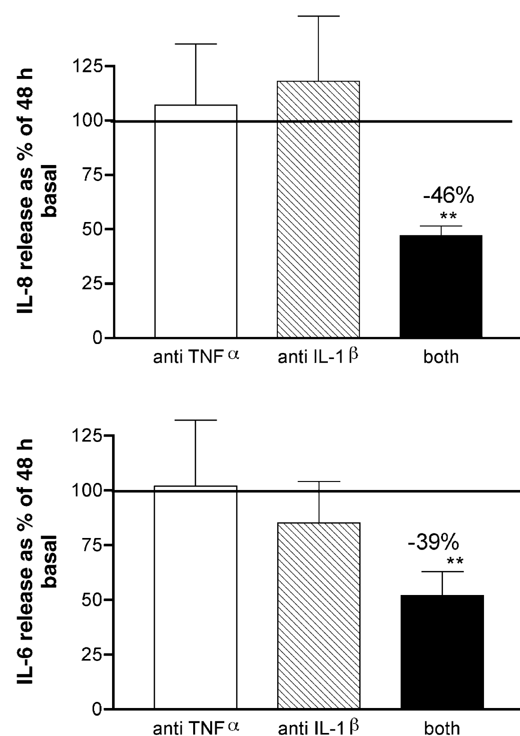

Fig. 3. Inhibition of endogenous TNFα and IL-1β release reduces IL-6 and IL-8 release by visceral adipose tissue explants. The minced tissue explants (1 g/2 ml of medium) were incubated for 5 min and then centrifuged to remove as much as possible of blood cells and tissue explants without enough lipid to float. The cut pieces of tissue (0.1 g/ml) were then incubated in duplicate for 48 h in the absence or presence of etanercept (which is a soluble human TNFα receptor), or the anti-human IL-1β antibody, or both at a concentration of 200 ng/ml. The values are shown as percentage ± S.E.M. of the 48 h control value for five paired experiments from as many individuals with an average body mass index of 48. The release over 48 h in the absence of the blockers was 800 pmol/g for IL-8 and 157 pmol/g for IL-6. The effect of both antagonists on IL-6 and IL-8 release were statistically significant ($p \geq 0.025$).

3.3. Effect of blocking endogenous TNFα or IL-1β release by adipose tissue explants in primary culture

We examined IL-6 and IL-8 release over a 48 h incubation of adipose tissue explants in the presence of etanercept (a recombinant protein that binds and inactivates human TNFα), a monoclonal anti-human IL-1β antibody (binds to and inactivates IL-1β), or both agents. The combination of both agents at a concentration of 200 ng/ml depressed the release of IL-6 by 39% and that of IL-8 over 48 h by 46% (Fig. 3). However, neither blocking agent alone significantly decreased IL-6 nor IL-8 release over 48 h.

We also examined the effect of the combination of etanercept and the anti-IL-1β antibody on IL-8 and IL-6 mRNA at shorter time periods. The data in Fig. 4a and b show that, in visceral adipose tissue, there was a 29% reduction in the increase over 1–3 h and a 42% reduction over 1 to 5 h in IL-8 mRNA due to the combination of the inhibitors of endogenous TNFα and IL-1β. The increase in IL-6 mRNA between 1 and 3 h was inhibited by 50% and that over 1–5 h by 55% by the combination of etanercept and the anti-human IL-1β blocking antibody.

3.4. Does actinomycin D affect up-regulation of IL-6 and IL-8 release as well as IL-6 and IL-8 mRNA accumulation in adipose tissue explants?

The transcriptional inhibitor actinomycin D reduced the rapid upregulation of IL-6 and IL-8 mRNA during the first 5 h after removal of adipose tissue from human donors (Fig. 4a and b). The upregulation of both IL-6 and IL-8 mRNA between 1 and 3 h of incubation was reduced by 67% and between 1 and 5 h of incubation by 75%. IL-6 and IL-8 release over 1–3 h was affected to only a small extent, but over 3–5 h of incubation IL-6 release was reduced by 30% and IL-8 release by 56% in the presence of actinomycin D (Fig. 4c and d).

3.5. What is the role of the NFκB pathway, p38 MAPK, ERK and JNK pathways in the up-regulation of IL-6 and IL-8 release?

The up-regulation of IL-8 release by human macrophages is dependent upon the NFκB pathway and influenced by the p38 MAPK, ERK and JNK pathways [26,27].

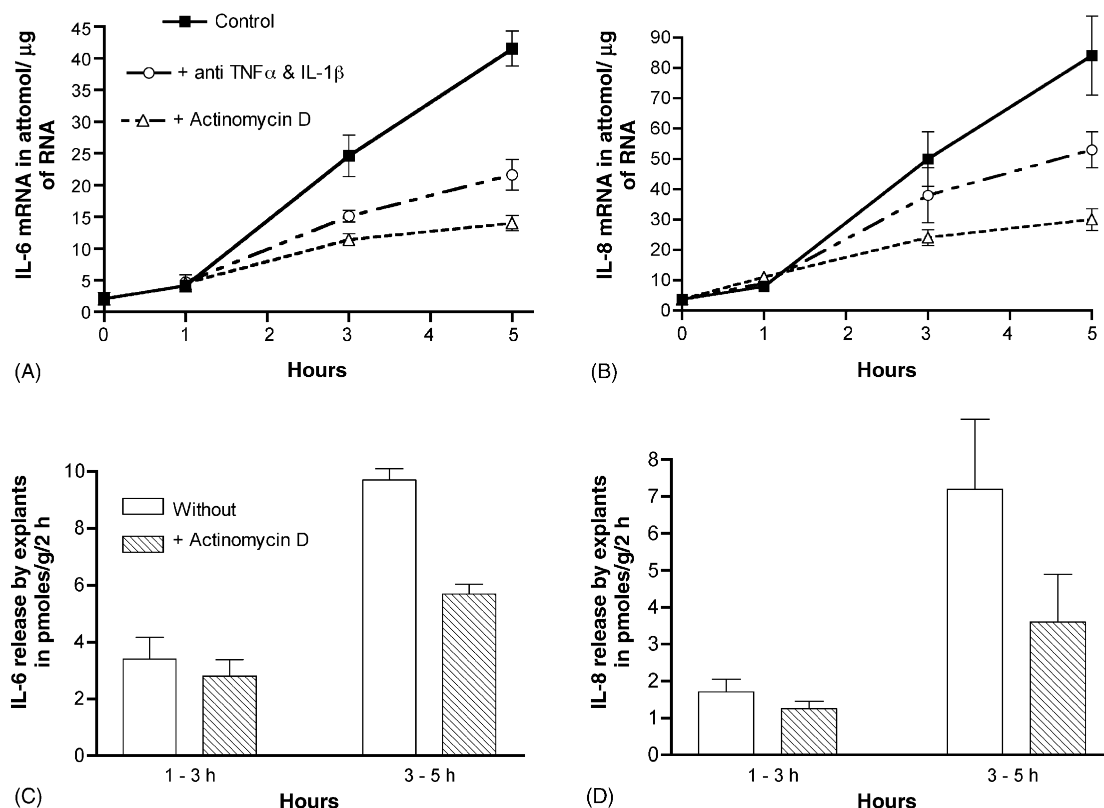

Fig. 4. Effect of actinomycin D or etanercept plus the anti-human IL-1β antibody on IL-6 and IL-8 release as well as IL-6 and IL-8 mRNA accumulation by explants of adipose tissue. Explants of adipose tissue were removed from the donors, minced and incubated for 0.5 h either without, with 0.1 µm actinomycin D or 1 µg/ml of etanercept, which is a soluble human TNFα receptor, plus 1 µg/ml of the anti-human IL-1β antibody. The explants were then subjected to brief centrifugation to remove the medium and resuspended in fresh medium either without, with actinomycin D or etanercept plus the anti-human IL-1β antibody and incubated for an additional 2 or 4 h. The values are shown as the mean ± S.E.M. of experiments using visceral adipose tissue from eight different individuals with an average body mass index of 48.

Table 1
Comparison of IL-8 and IL-6 formation as affected by inhibitors of the ERK, JNK, p38 MAPK and NFκB pathways

Inhibitor	IL-8 release inhibition (%)	IL-6 release inhibition (%)
+ERK inhibitor [UO-126]	18 ± 13	6 ± 12
+JNK inhibitor [JNK Inhibitor II]	0 ± 15	4 ± 10
+p38 MAPK inhibitor [SB 220025]	56 ± 8***	70 ± 10***
+NFκB inhibitor [quinazoline]	52 ± 6***	44 ± 7***
+ERK inhibitor + JNK inhibitor	23 ± 11	20 ± 3**
+ERK inhibitor + p38 MAPK inhibitor	77 ± 4***	79 ± 5***
+ERK inhibitor + NFκB inhibitor	61 ± 10***	52 ± 6***
+JNK inhibitor + p38 MAPK inhibitor	53 ± 9***	62 ± 5***
+JNK inhibitor + NFκB inhibitor	38 ± 12*	32 ± 20
+p38 MAPK inhibitor + NFκB inhibitor	86 ± 4***	82 ± 2***

Explants of visceral adipose tissue were incubated for 4 h in the presence of the following drugs each at a concentration of 1 μM: UO-126, an inhibitor of the ERK pathway; JNK inhibitor II, an inhibitor of the JNK pathway; SB220025, an inhibitor of the p38 MAPK pathway and a cell permeable quinazoline (4-phenoxyphenethyl 6-amino quinazoline], a NFκB inhibitor. The values are shown as the percentage inhibition ± S.E.M. of the paired differences due to the drugs for five experiments from as many individuals with an average BMI of 47. Basal IL-8 release over 4 h was 12 ± 3 pmol/g and IL-6 release was 11 ± 6 pmol/g. Significant effects of the drugs are indicated as follows.

* $p < 0.05$.

** $p < 0.01$.

*** $p < 0.005$.

Furthermore IL-6 mRNA degradation in human monocytes stimulated by lipopolysaccharide was markedly enhanced in the presence of an inhibitor of p38 MAPK pathway [28]. Therefore, we examined the effect of these pathways on IL-8 and IL6 release by explants of human adipose tissue using specific inhibitors of the NFκB, the p38 MAPK, ERK and JNK pathways at a concentration of 1 μM either alone or in combination with each of the other inhibitors (Table 1). The NFκB inhibitor and the p38 MAPK inhibitor decreased formation of IL-8 as well as that of IL-6. However, there were only small and statistically insignificant effects of the ERK or JNK inhibitors. In the presence of the ERK inhibitor plus the JNK inhibitor, no greater effect was seen than with either one on release of IL-8 or IL-6. In the presence of the ERK inhibitor, the p38 MAPK inhibitor had a greater effect than in its absence; while the JNK inhibitor did not enhance the action of the p38 MAPK inhibitor. Combining the NFκB inhibitor with the JNK inhibitor or the ERK inhibitor had no greater effect than the NFκB inhibitor alone. However, the combination of the p38 MAPK inhibitor and the NFκB inhibitor produced a greater inhibition than was seen with either alone.

The question of whether the inhibitors affected the net accumulation of IL-6 and IL-8 mRNA at the end of a 4 h incubation to the same extent as IL-6 and IL-8 release over 4 h was examined in the studies shown in Fig. 5. The effects of the p38 MAPK inhibitor, the NFκB inhibitor, or the combination of these inhibitors plus the ERK and JNK inhibitors were comparable to those seen with respect to release of both IL-6 and IL-8.

It is difficult to determine the true basal release in vivo of IL-6 and IL-8 by human visceral adipose tissue because of the marked upregulation seen after removal of the tissue. However, the unstimulated levels of IL-6 and IL-8 mRNA were approximately 2.4 and 2.6 amol, respectively, per microgram of RNA in adipose tissue immediately after removal from the donors (Fig. 4). After a 4-h incubation of

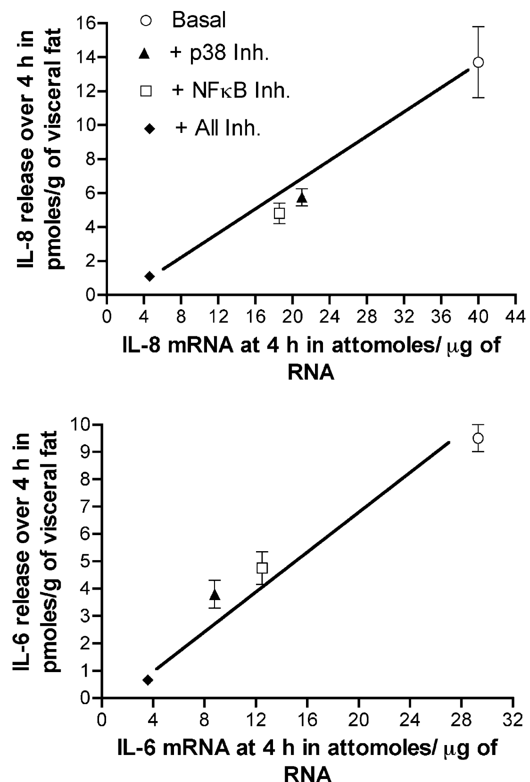

Fig. 5. Correlation between IL-8 as well as IL-6 release with IL-8 and IL-6 mRNA accumulation. Explants of visceral adipose tissue were removed from the donors, minced and incubated for 0.5 h. The explants were then subjected to brief centrifugation and suspended in fresh medium. The explants were incubated for 4 h in the presence of a 1 μM concentration of SB 220025 (an inhibitor of the p38 MAPK pathway), an inhibitor of the NFκB pathway (a cell permeable quinazoline compound also at 1 μM) or these drugs plus 1 μM UO-126 (an inhibitor of the ERK pathway) and JNK inhibitor II (an inhibitor of the JNK pathway) at 1 μM. The values for IL-8 or IL-6 release are shown as percentage ± S.E.M. for six experiments from as many different individuals with an average body mass index of 46. IL-6 and IL-8 mRNA were determined at the beginning and at the end of the 4 h incubation on the pooled tissues from the six experiments. IL-8 mRNA content at the start of the incubation was 2.4 amol/μg of RNA and that of IL-6 was 2.6 amol/μg of RNA.

adipose tissue in the presence of inhibitors of the p38 MAPK, NFκB, ERK and JNK, all at 1 μM, the mRNA values for IL-6 and IL-8 were 3.6 and 4.6. This was associated with release of 1.1 pmol/g of IL-8 and 0.7 pmol/g of IL-6 per 2 h. These values are probably close to the unstimulated value. Extrapolation of the linear relationship between RNA content and release shown in Fig. 5 suggests that at the zero time value for mRNA the release of IL-6 or IL-8 would be around 0.5 pmol/g per 4 h.

The enhanced release of IL-6 and IL-8 is biphasic and characterized by a slow initial rate of release during the first 2 as compared to the next 2-h of incubation (Fig. 6). Individually the NFκB or the p38 MAPK inhibitors reduced both phases of IL-8 and IL-6 release; while together they obliterated release during the first 2 h and markedly reduced the slow release phase. These data indicate that while the effect of actinomycin D on IL-6 and IL-8 release is delayed in onset (Fig. 4), this is not the case for the inhibitors of the p38 MAPK and NFκB pathways.

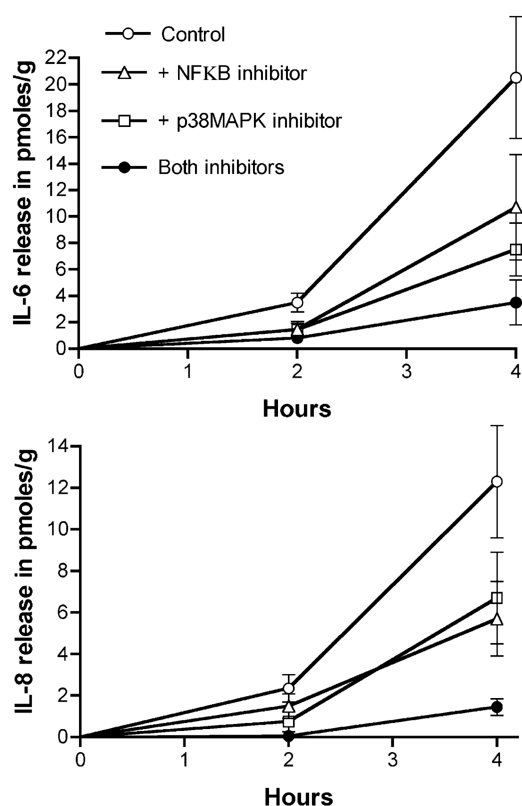

Fig. 6. Comparison at 2 and 4 h of IL-8 and IL-6 release in the absence or presence of an inhibitor of NFκB, an inhibitor of p38 MAPK or both inhibitors. Explants of visceral adipose tissue were removed from the donors, minced and incubated for 10 min. The explants were then subjected to brief centrifugation and suspended in fresh medium. The explants were incubated for 2 or 4 h in the presence of 1 μM SB 220025 (an inhibitor of the p38 MAPK pathway) or 1 μM NFκB inhibitor (a cell permeable quinazoline compound) or both drugs. The values for IL-8 or IL-6 release are the mean ± S.E.M. for five experiments from as many different individuals with an average body mass index of 46.

4. Discussion

Ruan et al. [4] suggested that the digestion of murine adipose tissue with collagenase resulted in an upregulation of the release of IL-6 and other adipokines by adipocytes. However, in their report they also said that "secretion of TNFα and IL-6 occur at similar levels even if collagenase is not added to the disrupted adipose tissue prior to incubation" [4]. When we examined IL-6 mRNA up-regulation in explants of human visceral adipose tissue, we found a lag period of at least 1 h before any up-regulation could be seen. Furthermore, this up-regulation was primarily in the non-fat cells of adipose tissue and the IL-6 mRNA content per unit of total RNA in the adipose tissue matrix after a 2 h collagenase digestion was comparable to that in intact adipose tissue.

It was not possible to determine whether collagenase digestion rapidly upregulated the IL-6 mRNA content of adipocytes prior to 3 h after removal of adipose tissue from humans because the first point that adipocyte mRNA could be obtained was at 3 h. However, there was no further increase in IL-6 mRNA in adipocytes at 27 or 51 h suggesting that the amount of IL-6 mRNA in adipocytes was not up-regulated. In contrast, we did see an up-regulation of IL-8 mRNA in adipocytes at 27 and 51 h as compared to 3 h. These data suggest that IL-8 is upregulated in human adipose tissue explants to a greater extent than IL-6, which is in agreement with our studies on their release over 4, 24 and 48 h [10].

We previously reported an upregulation of IL-6 and IL-8 release by bacterial lipopolysaccharide as well as an inhibition by dexamethasone of IL-6 and IL-8 release over a 48 h incubation of human subcutaneous adipose tissue explants [29]. Similar results have been reported using human fibroblasts and monocytes [30–32] where dexamethasone inhibited IL-6 formation while lipopolysaccharide stimulated IL-6 formation.

Bruun et al. [33] found that the addition of TNFα or IL-1β to explants of subcutaneous adipose tissue from the abdominal region of women with an average body mass index of 25 resulted in an enhanced release of IL-8. Flower et al. [34] reported that IL-1β enhanced the release of IL-6 by human adipocytes and peripheral blood cells. These data suggested that TNFα and/or IL-1β could be involved in the up-regulation of IL-6 and IL-8 release that we observed in human visceral adipose tissue. Therefore, it was not surprising that blocking the action of endogenous IL-1β and TNFα release by cut explants of adipose tissue reduced the upregulation of IL-6 and IL-8. The TNFα antagonist we used was the recombinant protein etanercept that binds TNFα and is used clinically in the treatment of rheumatoid arthritis [35]. We used a monoclonal blocking antibody against human IL-1β for the in vitro studies which should have effects similar to those of a human IL-1β receptor antagonist that is also effective in the treatment of rheumatoid arthritis [36]. Park et al. [37]

have shown that, in cultured pleural mesothelial cells, the induction of IL-8 formation by conditioned media from M. tuberculosis-infected pleural macrophages was almost completely blocked by the combination of a soluble IL-1β receptor antagonist and an antibody against TNFα. In our experiments, using antagonists of IL-1β and TNFα, IL-8 as well as IL-6 formation was reduced by 50% suggesting that other factors are involved as well. Since the up-regulation of IL-8 and IL-6 mRNA as well as their release over 4 h in one cut piece of adipose tissue was similar to that seen to minced pieces of adipose tissue, it appears that these effects do not require mincing of the tissue.

The regulation of IL-6 formation in murine bone marrow-derived mast cells by IL-1β involves enhanced transcription of IL-6 mRNA as well as mRNA for a protein that stabilizes IL-6 mRNA [38]. The addition of IL-1β results in induced formation of a protein that stabilizes IL-6 mRNA in human peritoneal mesothelial cells [39]. The effect of IL-1β can be mimicked in lung H292 cells by cycloheximide that increases IL-6 mRNA by preventing formation of the protein that degrades the mRNA [40]. Our studies using explants of human visceral adipose tissue indicate a lag period of at least 1 h before an elevated level of IL-6 mRNA is seen. Actinomcyin D reduced the increases seen in IL-6 mRNA at 3 and 5 h in visceral adipose tissue explants by 69 and 75%, respectively, which suggests that the increase in IL-6 mRNA is in part due to increased transcription of mRNA in visceral adipose tissue. However, mRNA stability is also important since it has been shown IL-6 mRNA decay is accelerated in human monocytes by a p38 MAPK inhibitor [28]. We found that, in fact, a p38 MAPK inhibitor along with a NFKB inhibitor reduced IL-6 and IL-8 release by visceral adipose tissue explants over 4 h incubation far more rapidly and to a much greater extent than did actinomycin D.

Under basal conditions, IL-8 formation by macrophages is difficult to detect but its formation is rapidly enhanced by three different mechanisms [26]. One involves derepression of the gene promoter, another transcriptional activation of the gene and the third stabilization of the mRNA. Hoffman et al. [26] concluded that p38 MAPK, JNK kinase, ERK and NFκB are all involved in the IL-8 upregulation in macrophages. We found somewhat different results in human visceral adipose tissue explants where over a 4-h incubation the formation of IL-8 as well as that of IL-6 was primarily regulated by the p38 MAPK and NFκB pathways.

Our evidence for the involvement of the p38 MAPK and NFκB pathways in IL-6 and IL-8 release by adipose tissue is based on the use of inhibitors of these pathways. The effects of the drugs could be due to toxic effects but over a 48 h incubation of visceral adipose tissue explants in the presence of a 1 μM concentration of the ERK, plus the JNK inhibitor, plus the p38 MAPK and the NFκB inhibitors IL-8 release was inhibited by 81% while that of TGF-β1 was not significantly inhibited ($-4 \pm 5\%$ as the mean change in four paired experiments). In the same experiments lipolysis was also unaffected ($-1 \pm 7\%$). These data suggest that the drugs are not having non-specific effects.

The upregulation of IL-6 and IL-8 release is not due to cutting of adipose tissue but appears to involve the release of endogenous TNFα and IL-1β and other regulators of NFκB and the p38 MAPK pathways. It was surprising that there was the same upregulation of IL-6 and IL-8 release in one cut piece of fat (200 mg) incubated in medium for 4 h as in many smaller pieces (5–20 mg). It was anticipated that there would be relative hypoxia in one cut pieces of tissue. However, after severing the blood supply to human visceral adipose tissue it is brought as one or two larger pieces of fat (10–20 g) to the laboratory at 37° C and 15–30 min elapse before it is cut into smaller pieces and put in medium. Relative hypoxia during this time could be involved in the upregulation of IL-6 and IL-8 mRNA in adipose tissue.

It has been suggested that macrophage accumulation in adipose tissue accounts for much of the enhanced release of adipokines other than leptin and adiponectin that is seen in obesity [41,42]. These finding are compatible with our hypothesis that the release of adipokines such as IL-6 and IL-8 by human adipose tissue is primarily by cells other than adipocytes. Whether macrophages account for the majority of the adipokine release by explants of human adipose tissue remains to be demonstrated. Our data do demonstrate that if macrophages account for most of the release of IL-6 and IL-8 by human adipose tissue, they are not released from the adipose tissue matrix during collagenase digestion of human fat. Further studies are needed to elucidate the nature of the non-fat cells found in adipose tissue that are responsible for the release of adipokines such as IL-6 and IL-8 and the mechanisms involved in this rapid upregulation.

In conclusion, IL-6 and IL-8 mRNA expression are rapidly upregulated within 3 h after removal and in vitro incubation of visceral adipose tissue from humans. Whether transient hypoxia plays a role is unclear but at least half of the upregulation appears to be due to release of TNFα and IL-1β.

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