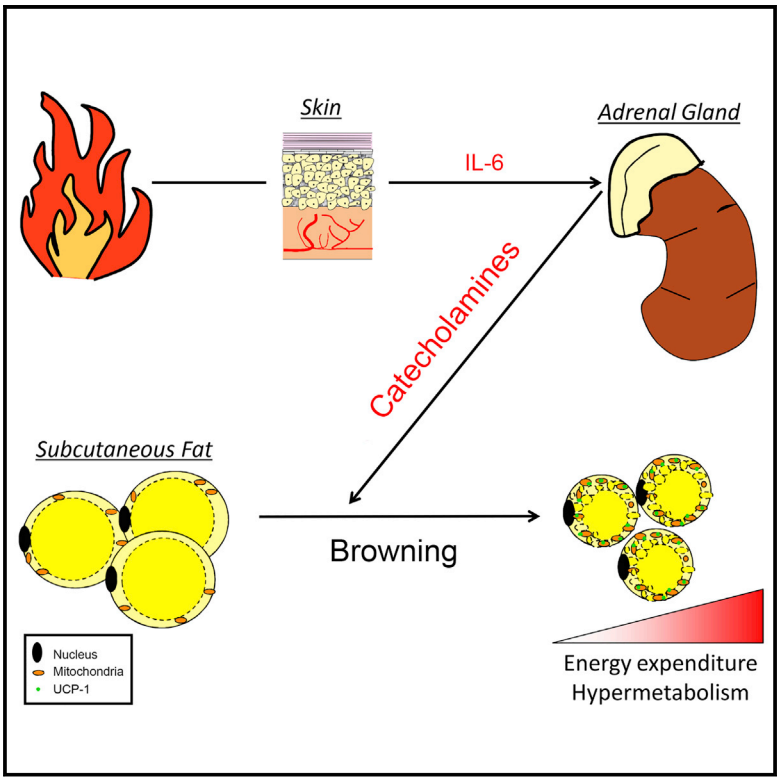
Cell Reports

Burn Induces Browning of the Subcutaneous White Adipose Tissue in Mice and Humans

Graphical Abstract



Authors

David Patsouris, Peter Qi, Abdikarim Abdullahi, ..., Alexandra Parousis, Saeid Amini-Nik, Marc G. Jeschke

Correspondence

marc.jeschke@sunnybrook.ca

In Brief

Severe trauma such as burn injury is followed by a hypermetabolic state that is characterized by an elevation in energy expenditure and insulin resistance. Patsouris et al. show that severe burn injury results in browning of the subcutaneous fat; this may explain why these patients develop hypermetabolism.

Highlights

- Burn injury results in browning of the subcutaneous fat in rodents and humans
- Browning occurs beyond or after 10 days post-burn injury in humans
- Markers of browning are reduced by the beta-blocker propranolol
- IL-6 is required for browning in mice post-burn injury

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Cell Reports
Report

Burn Induces Browning of the Subcutaneous White Adipose Tissue in Mice and Humans

David Patsouris,[3] Peter Qi,[3] Abdikarim Abdullahi,[3] Mile Stanojcic,[3] Peter Chen,[3] Alexandra Parousis,[3] Saeid Amini-Nik,[2,3] and Marc G. Jeschke[1,2,3,*]
[1]Ross Tilley Burn Centre, Sunnybrook Health Sciences Centre, Sunnybrook Research Institute, Toronto, ON M4N 3M5, Canada
[2]Sunnybrook Research Institute, Toronto, ON M4N 3M5, Canada
[3]Department of Surgery, Division of Plastic Surgery, Department of Immunology, University of Toronto, Toronto, ON M5S 1A1, Canada
*Correspondence: marc.jeschke@sunnybrook.ca
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SUMMARY

Burn is accompanied by long-lasting immunometabolic alterations referred to as hypermetabolism that are characterized by a considerable increase in resting energy expenditure and substantial whole-body catabolism. In burned patients, the length and magnitude of the hypermetabolic state is the highest of all patients and associated with profoundly increased morbidity and mortality. Unfortunately, the mechanisms involved in hypermetabolism are essentially unknown. We hypothesized that the adipose tissue plays a central role for the induction and persistence of hypermetabolism post-burn injury. Here, we show that burn induces a switch in the phenotype of the subcutaneous fat from white to beige, with associated characteristics such as increased mitochondrial mass and UCP1 expression. Our results further demonstrate the significant role of catecholamines and interleukin-6 in this process. We conclude that subcutaneous fat remodeling and browning represent an underlying mechanism that explains the elevated energy expenditure in burn-induced hypermetabolism.

INTRODUCTION

Hypermetabolism in burned patients is reflected by a biphasic elevation of REE that lasts at least up to 36 months post-burn and extends in parallel with the levels of stress hormones (Jeschke et al., 2011; Kraft et al., 2011). Hypermetabolism is associated with other known comorbidities of burn injury, such as insulin resistance, liver steatosis, massive lipid and protein catabolism, and hyperinflammation (Williams et al., 2009). The extent and persistence of this substantial hypermetabolic catabolic response is unique for burn patients and, despite its importance, it is currently unclear whether and how these symptoms are interconnected.

Uncoupling mitochondrial ATP synthesis is a well-established mechanism that elevates energy expenditure. Three uncoupling proteins (UCPs) have been described to date. UCP1 is the only exclusively expressed in adipose-specific depots, in particular, the brown adipose tissue (Wu et al., 2013). UCP2 is found in many tissues, and UCP3 is considered mostly specific to skeletal muscle (Brand and Esteves, 2005). However, UCP1 is quite unique as it is the only UCP that is considered involved in uncoupling- mediated energy expenditure. Accordingly, increasing UCP1 activity has been considered an attractive strategy to combat obesity. Whereas the existence of a bona fide functional brown adipose tissue and its contribution to overall energy homeostasis in adult humans are still subject to debate, experts acknowledge the presence of an intermediary type of adipose tissue between the white and the brown adipose tissue, which has been named beige or brite adipose tissue (Seale and Lazar, 2009; Sharp et al., 2012; Yoneshiro et al., 2013). Interestingly, the subcutaneous fat is capable to switch from a white to a brite phenotype, in a process referred to as ''browning'' (Cohen et al., 2014; Harms and Seale, 2013; Shabalina et al., 2013). Little is known about browning in burn patients, but based on the pathophysiology of burns and its persistent effect, we hypothesized that browning is part of the response after burn.

Additionally, we attempted to determine the mechanisms by which browning is induced. Catecholamines are the most-described drivers of the phenotypic switch from white to beige (Nguyen et al., 2011). Furthermore, catecholamines are chronically elevated in burn patients and their concentration positively correlates with severity of hypermetabolic symptoms (Williams et al., 2009; Wilmore et al., 1974). Moreover, propranolol, a non-selective beta- receptor blocker, has been shown to decrease hypermetabolic catabolism, as well as to attenuate burn-induced increase in energy expenditure (Herndon et al., 2012; Williams et al., 2009), indicating an important role for catecholamines during the process of browning. Consequently, our goal was to investigate whether burn induces a phenotypic switch from white to beige in the subcutaneous fat tissue and potential mechanisms implementing animal models but also burn patients.

RESULTS

Burn Induces Browning of Mice Inguinal Fat

First, we performed histological analyses of the epididymal white adipose tissue (eWAT), interscapular brown adipose tissue

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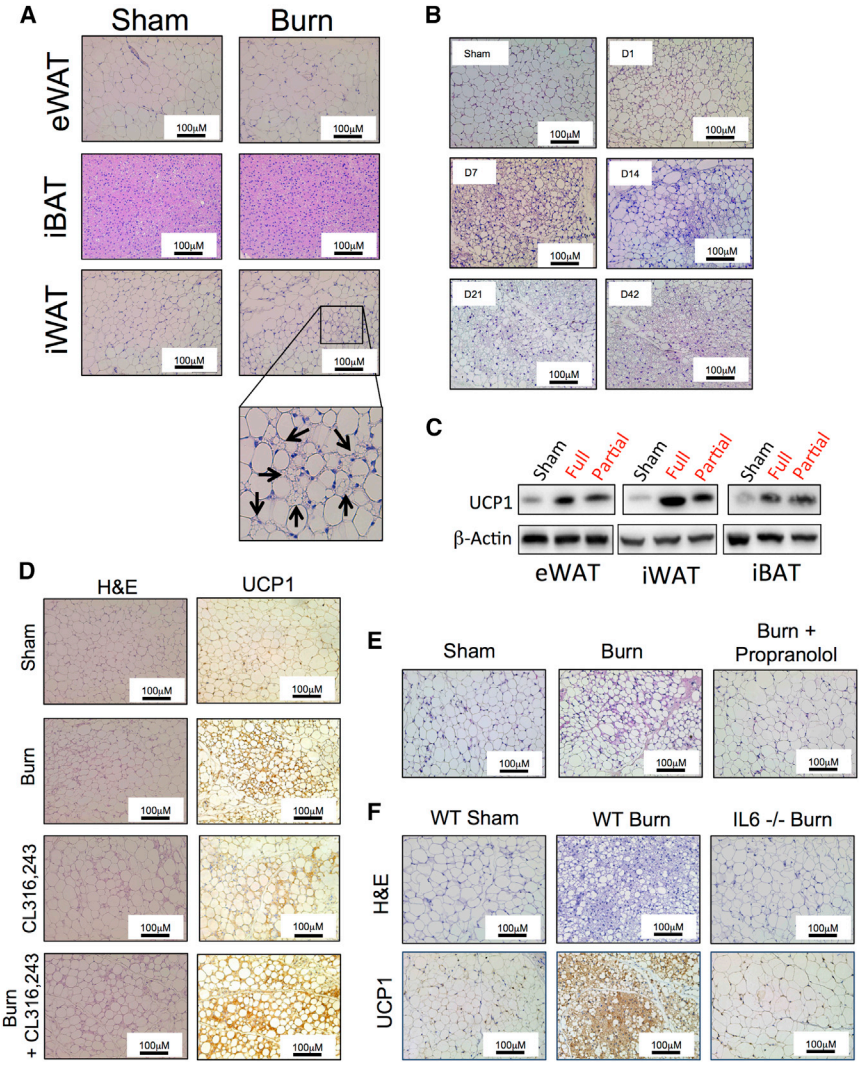

Figure 1. Burn-Induced Adipose Tissue Remodeling and Browning

We collected epididymal fat (eWAT), interscapular brown fat (iBAT), and inguinal fat (iWAT) of mice sham or 2 days post-burn (30% TBSA; n = 3).

(A) H&E staining is shown.

(B) H&E of iWAT of sham mice or mice that had been burned (30% TBSA) for 1, 7, 14, 21, or 42 days.

(C) Western blot of UCP1 in epididymal white adipose tissue (eWAT), inguinal white adipose tissue (iWAT), and interscapular brown adipose tissue (iBAT) of mice sham treated or burned to full or partial thickness (24 hr post-burn; n = 3).

(D) H&E staining and UCP1 staining of iWAT of sham mice compared to burned mice (24 hr post-burn) injected with CL316,243 (1 mg/kg) or burned and injected with CL316,243. n = 3–5 per group.

(E) Mice were burned and received i.p. injection of propranolol daily or sham (n = 5). The iWAT was collected 48 hr post-burn and stained with H&E.

(F) Wild-type (WT) and IL-6 knockout (IL6KO) were burned, and 72 hr later, the inguinal fat was collected and either stained with H&E or stained with UCP1 antibody (n = 3).

(iBAT), and inguinal white adipose tissue (iWAT) in control (sham) and burned mice (2 days post-burn; 30% TBSA). As illustrated in Figure 1A, no striking morphological differences could be observed in eWAT and iBAT. However, we noticed the presence of multilocular adipocytes in the iWAT of burned mice, which was not observed in sham mice. This tissue remodeling was detected as early as 24 hr post-burn and persisted for at least 42 days post-burn (Figure 1B). The presence of multilocular adipocytes is characteristic of beige adipose tissue, which suggests that burn triggers adipocytes to transdifferentiate from white to beige. Consequently, we proceeded to the quantification of UCP1, a specific marker for fat browning, in different fat depots. As shown in Figure 1C, UCP1 was strongly induced by burn in epididymal fat (eWAT), inguinal fat (iWAT), and iBAT. Interestingly, this upregulation was more pronounced following full thickness burn compared to partial burn in eWAT and iWAT. However, as shown in Figure S1, UCP1 expression post-burn was higher in the two fat depots, namely the iWAT and the iBAT compared to eWAT. Subsequently, we proceeded to the immuno-staining of the iWAT with the fat-browning-specific marker, UCP1. In addition to burn, we also injected mice with the β3-specific adrenergic agonist CL316,243, with and without burn injury (Weyer et al., 1999). We confirmed that burn injury induced iWAT remodeling within 24 hr and UCP1 staining co-localized with multilocular adipocytes (Figure 1D). Furthermore, browning of the tissue induced by burn was comparable to the effect of the adipose-specific β3-agonist (Barbatelli et al., 2010). The elevated energy expenditure following burn and/or administration of CL316,243 was supported by the significant decrease in eWAT adipocyte sizes (Figures S2A and S2B; Ghorbani and Himms-Hagen, 1997). Macrophages have been reported to mediate iWAT browning in response to cold exposure (Nguyen et al., 2011). However, presently, macrophages depletion with clodronate did not prevent burn-induced iWAT browning (Figures S3A and S3B). Alternatively, the unspecific β-blocker propranolol decreases energy expenditure in burn patients. Furthermore, catecholamines are elevated post-burn and are known to stimulate iWAT browning (Herndon et al., 2012; Williams et al., 2009; Nguyen et al., 2011). Consequently, we tested whether browning of the iWAT caused by burn was dependent on β adrenoceptor signaling. As illustrated in Figure 1E, injection of the β-blocker propranolol was able to interfere with the burn-induced remodeling of the iWAT caused by burn in mice. In addition, IL-6 is a cytokine that is robustly secreted post-burn (Williams et al., 2009). Furthermore, this cytokine is known to stimulate energy expenditure through the CNS (Wallenius et al., 2002) and it was recently reported

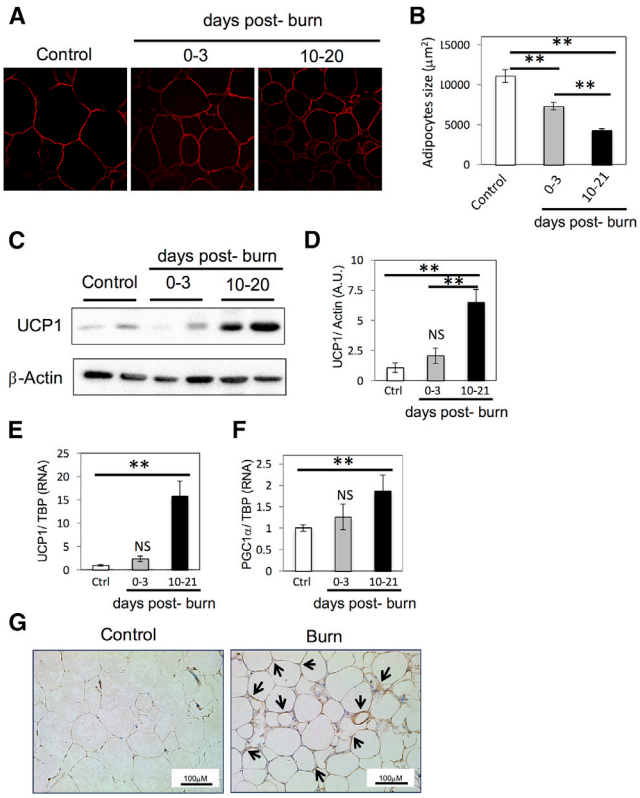

Figure 2. Reduced Adipocyte Size and Delayed Onset of Browning in Burned Patients

(A) Perilipin staining of subcutaneous fat extracted from the wounded area in human patients at the indicated days post-burn. Control subcutaneous fat from healthy patients is shown for reference (n = 5 per group).

(B) The representation of the surface area of the corresponding adipocytes is shown.

(C) UCP1 was quantified by western blot in the subcutaneous fat collected post-surgery in burned patients.

(D) The normalized expression of UCP1 is shown (n = 8 per group).

(E and F) UCP1 (E) and PGC1α (F) expression was quantified by qPCR and normalized to the housekeeping gene TBP (n = 8 per group).

(G) Fat tissues from healthy control or burned patients (>10 days post-burn) were stained with a UCP1 antibody.

** indicates $p < 0.05$.

that it is also involved in the browning of the adipose tissue that occurs in response to specific stimuli (Buzelle et al., 2015; Knudsen et al., 2014; Petruzzelli et al., 2014). We hence decided to evaluate the contribution of IL-6 in burn-induced iWAT browning. As shown in Figure 1F, there were no signs of tissue remodeling or induction of UCP1 expression in burned IL-6−/− mice.

Burn Induces Browning Markers in Humans

We then decided to test whether a similar switch from white to beige occurred in human fat tissue and performed histological analyses of human subcutaneous fat tissues collected from patients during burn surgery. As shown in Figures 2A and 2B, staining of perilipin demonstrated that burn induces a very marked decrease in the adipocytes size. H&E staining of the fat confirmed the decreased size of adipocytes in patients 10–21 days post-burn, demonstrated by a predominance of small adipocytes, whereas adipocytes were of medium size in patients 0–3 days post-burn (Figure S4A). Next, we quantified the expression of UCP1 in protein extracts from burn patients' subcutaneous fat. As shown in Figures 2C and 2D, there was a marked increase (~7-fold) in the expression of this brown adipose tissue marker in fat that was excised during the patients last surgery (10 days to 3 weeks post-burn). This upregulation was not visible on fat collected during the first surgery (0–3 days post-burn). We next quantified with qPCR the expression of UCP1 and PGC1α, a master driver coordinating the genetic program involved in beige fat formation. As shown in Figures 2E and 2F, the expression of UCP1 and PGC1α was very significantly stimulated in the subcutaneous fat of patients collected during last surgery. We also quantified by qPCR the expression of the β3-adrenoceptor, but no significant change could be detected at early or late time point post-burn. Finally, we performed an immuno-staining of subcutaneous fat tissues from burn patients with UCP1. We noticed that UCP1 was indeed expressed by adipocytes from burned patients, with a higher expression present on smaller adipocytes (Figure 2G).

Burn Increases Mitochondrial Mass in Subcutaneous Fat

In addition to the significant expression of UCP1, beige fat is characterized by increased mitochondrial mass (Harms and Seale, 2013). Therefore, we performed an immunofluorescence analysis of the tissue with the mitochondrial mass marker VDAC1 (Porin). As shown in Figure 3A, we observed a higher intensity of staining for VDAC1 in subcutaneous tissues from patients collected during last surgery. Western blotting confirmed higher expression of VDAC1 (~2-fold) in human s.c. fat (Figure 3C). We also performed an analysis of fat tissues with TEM. Whereas mitochondria were scarce in control fat tissues (Figure 3D), we observed that adipocytes in the fat collected from burned patients during their last surgery were enriched in mitochondria (Figures 3E and 3F). Moreover, we observed the presence of multiple small lipid droplets in the cytosol of adipocytes from fat tissues of burned patients.

Mechanisms Involved in Burn-Induced Subcutaneous Adipose Tissue Browning in Humans

The release of catecholamines by the sympathetic nervous system is a well-characterized driver for beige fat formation in the context of non-shivering thermogenesis. Severe thermal injury is accompanied by long- lasting elevated plasma catecholamine concentration. Furthermore, the beta- blocker, propranolol, decreases hypermetabolic symptoms including resting energy expenditure in burned patients (Herndon et al., 2012; Williams et al., 2009). As shown in Figure 4A, propranolol administration lowered patients' MREE. Unfortunately, despite the signal indicating the effect of propranolol on MREE, due to the low number of patients involved in this cohort (n = 6), the results did not reach statistical significance. Next, we quantified the expression of UCP1 and the mitochondrial complex IV in the subcutaneous fat tissues of patients burned that were

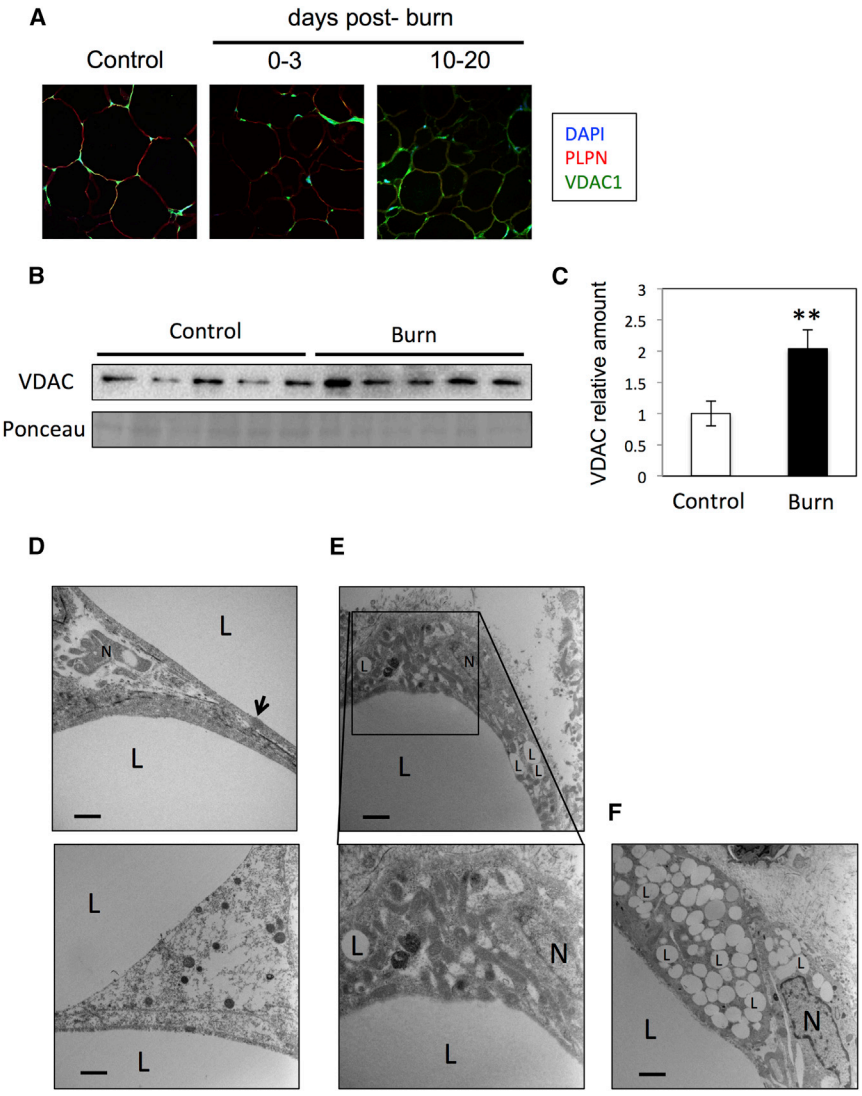

Figure 3. Increased Mitochondrial Expression in Adipose Tissue Post-burn

(A) Human subcutaneous tissue from healthy control or burned patients was collected and stained for VDAC1, perilipin, and DAPI.

(B) VDAC1 expression was determined by western blot in the subcutaneous fat from healthy control or burned patients (>10 days post-burn).

(C) VDAC1 expression was normalized to an unspecific band. ** indicates $p < 0.05$.

(D–F) Analysis of fat from healthy patients performed by TEM (D), whereas (E) represents the fat tissues from patients burned at least 10 days earlier. L, lipid droplet; N, nuclei. Arrow in (D) indicates a mitochondrion. The lower panel in (E) and (F) is an enlargement of the region representing the mitochondrial enrichment in the cytosol of adipocytes from burned patients. n = 3 per group. The bar scale at the bottom left corner represents 500 nm in (D) and (E) and 125 nm in (F).

treated with propranolol (Figure 4A). As shown in Figure 4B, the increased expression of UCP1 and COX IV caused by burn was significantly prevented by treatment with propranolol. Our results obtained in mice (Figure 1F) suggest that IL-6 is connected with burn-induced increase of energy expenditure. Therefore, we also measured the resting energy expenditure (MREE) of burned patients and their plasma levels of IL-6. As shown in Figure 4C, we observed that variations in IL-6 levels paralleled with that of energy expenditure in burned patients. Finally, we performed a correlation analysis between IL-6 and MREE. Figure 4D shows that indeed there is a significant positive correlation between IL-6 and MREE in burned patients ($p = 0.037$). In contrast, other cytokines not related to browning such as IL-8 and TNF-α significantly were not correlated with MREE (Figures S4B and S4C). These observations support the hypothesis that browning caused by burn and subsequent increase energy expenditure may involve the contribution of this cytokine.

DISCUSSION

Our results demonstrate that severe thermal injury induces subcutaneous fat browning. This modification is confirmed at the morphological, cellular, and molecular levels, extending from mouse to human. Increasing energy expenditure through the recruitment of beige fat and activation of the brown adipose tissue in human represents a novel and exciting strategy for the treatment of obesity and associated diseases. This interest has led to the identification of several endogenous molecules reported to lead to this activation. Among them, catecholamines released by the CNS through the direct activation of the β3-adrenoceptor are considered major stimulating factors (Cypess et al., 2015). Yet other factors such as IL-6 or lactate have also been reported to stimulate this pathway (Carrière et al., 2014; Petruzzelli et al., 2014). Interestingly, these three factors are all elevated in burn patients (Williams et al., 2009). Thus far, it is not clear whether the β3-adrenoceptor receptor and the IL-6 cytokines pathways are interconnected or independent. However, a recent finding indicates that systemic inflammation and IL-6 mediates white adipose tissue browning, which is further responsible for high-energy expenditure and catabolism in cancer-associated cachexia (Petruzzelli et al., 2014). Furthermore, IL-6 knockout mice exhibit enlargement of the subcutaneous, inguinal fat depots, which is counteracted by intracerebroventricular injection of IL-6 (Wallenius et al., 2002). The fact that this property was mediated by increased energy expenditure suggests that IL-6 mediates browning of the adipose tissue in an indirect manner, through the CNS. As the sympathetic nervous system controls adrenal catecholamine secretion, it is plausible that IL-6 acts upstream of the catecholamine-signaling pathway, leading to

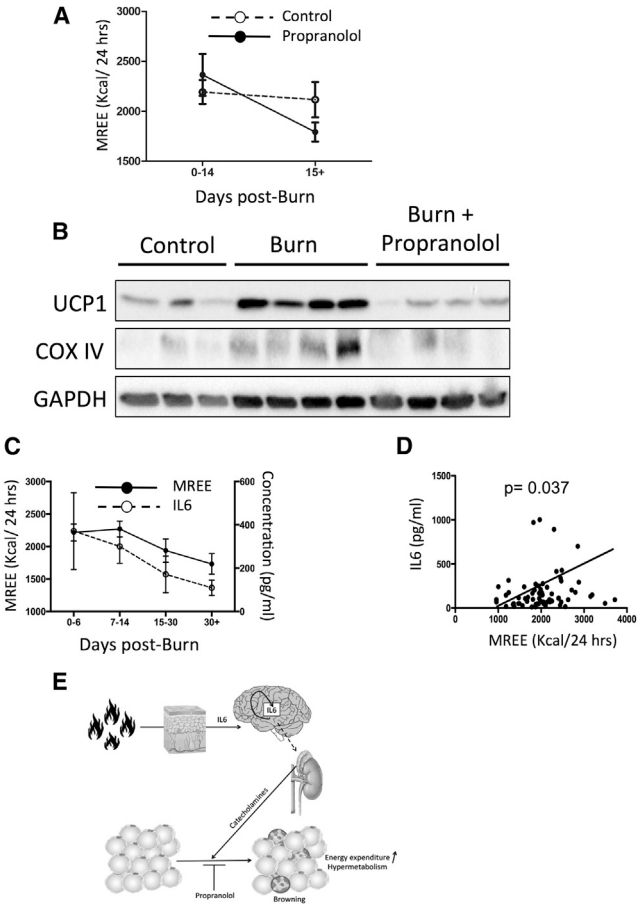

Figure 4. The Antagonistic Effects of Propranolol and IL-6 in Adipose Tissue Post-burn

(A) Average measured resting energy expenditure (MREE) from patients untreated or before and after daily treatment with propranolol (n = 6).

(B) Western blots representing the expression of UCP1 and COX IV in the subcutaneous fat tissue of healthy control or burned patients untreated or treated with propranolol (>10 days post-burn).

(C) The graph illustrates the MREE of burned patients in parallel with average IL-6 plasma concentrations over time post-burn.

(D) Correlation of patients' plasma IL-6 concentrations with patients MREE.

(E) Burn induces IL-6 expression, which targets the CNS, translating in the stimulation of catecholamines releases by the adrenal glands. Elevated catecholamines induce subcutaneous fat browning and subsequent elevation of the energy expenditure, characteristic of hypermetabolism.

the browning of the white adipose tissue. The fact that the CNS plays a key role in hypermetabolism is supported by the observation that brain trauma is a well-established cause of hypermetabolism (Foley et al., 2008). In patients, we found that propranolol treatment reduced MREE. However, blockade of IL-6, in particular in the CNS, may provide an alternative strategy to treat hypermetabolic symptoms in burned patients, possibly with greater and broader extent.

Although the remodeling of the subcutaneous fat tissue is observed as early as 24 hr post-burn in mice, the same process takes longer in human patients. Presently, the explanation for such difference is not clear. However, trauma is characterized by a biphasic metabolic response. The early phase also referred as "ebb" phase lasts from 24 to 72 hr post-trauma and is characterized by decreased energy expenditure. The "flow" phase that follows is when hypermetabolism is observed and energy expenditure is increased (Breznock, 1980). Therefore, tissues collected from patients on first surgery correspond to tissues collected during the ebb phase, whereas tissues collected subsequently are collected from patients in the flow phase and experiencing an increase in energy expenditure. These two opposite phases could explain the absence of browning markers when fat tissue is collected within 3 days post-burn.

A recent report established that burn induces activation of the iBAT and corresponding UCP1 expression in rats (Yo et al., 2013). Confirming this observation, we noticed a higher expression of UCP1 in the iBAT of burned mice (Figure 1C). However, our mice are housed at ambient temperature; therefore, the brown adipose tissue may already be in an activated state, explaining why we did not observe any significant morphological changes in the iBAT (Figure 1A). Interestingly, we also observed that UCP1 was also very significantly expressed and induced by burn in the skin. Whereas UCP1 is usually considered specific for adipose depots, UCP1 expression has also been reported in the skin (Mori et al., 2008). However, the skin is a heterogeneous tissue, which also includes a thin layer of adipocytes. Furthermore, it should be noted that burn alters skin phenotype and in particular may decrease the protection against cold, which is known to induce browning of the subcutaneous fat. Therefore, future work should determine in which cell types UCP1 is expressed post-burn in the skin tissue and what is the overall contribution of this tissue in burn-induced subcutaneous fat browning.

It may seem paradoxical that both cold exposure and burn induce a similar remodeling of the subcutaneous fat tissue. However, these two external stresses lead to an adaptive response that shares several parameters. First, both lead to the increased secretion of catecholamines. Second, they both stimulate lipolysis and subsequent release of free fatty acids in the circulation (Williams et al., 2009). These events possibly play a great role in the activation of energy expenditure post-burn as free fatty acids are required for full activation of UCP1 (Fedorenko et al., 2012).

These findings suggest that thermal injury induces a profound remodeling of the subcutaneous fat tissue. The resulting switch from white to beige fat that occurs post-burn may represent the underlying mechanisms governing the elevation of energy expenditure experienced by burn patients. The observation that propranolol decreases fat browning caused by burn as well as MREE supports this hypothesis. Furthermore, we illustrate that IL-6 is involved in the pathway that leads to these modifications and blockade of this cytokine may represent an interesting strategy to treat hypermetabolism in burn patients. It would be of particular interest to test whether similar alterations occur in other forms of trauma, but future work is warranted to fully answer this question.

EXPERIMENTAL PROCEDURES

Human Samples

Patients admitted to the Ross Tilley Burn Centre at Sunnybrook Hospital or patients undergoing elective surgery were consented pre-operatively for tissue

collection. Approval for our study was obtained from the Research Ethics Board at Sunnybrook Hospital. We enrolled 20 severely burned adults (46.3 ± 3.9 years old; 14 males and 6 females) with burns encompassing 48% ± 3.9% of their total body surface area (TBSA). Fat obtained at first OR (less than 3 days) and last OR (greater than 10 days) from patients were immediately transferred to the laboratory and either frozen at −80°C until further analysis or transferred in fixative. MREE was measured as previously described (Jeschke et al., 2011). Propranolol was administrated according to standard dosing and protocols in order to decrease heart rate below 100 bpm. IL-6 in plasma was determined using a multiplex platform (Millipore) in accordance with manufacturer's protocol.

Mice Model

Male C57BL/6 mice (Jackson Laboratory) were housed at ambient temperature and cared in accordance with the Guide for the Care and Use of Laboratory Animals. All procedures performed in this study were approved by the Sunnybrook Research Institute Animal Care Committee. Unless specified otherwise, full thickness (30% TBSA) was used to burn the mice. Full thickness was achieved in immersing the back of the mice (30% TBSA) at 98°C for 10 s, whereas partial thickness (30% TBSA) was achieved at 60°C for 18 s (Bayliss et al., 2014). Burned mice were subsequently housed individually in sterile cages and fed ad libitum until sacrifice. IL-6 knockout mice were obtained from Jackson Laboratories. CL316,243 (1 mg/kg/day) and propranolol (20 mg/kg/day) were obtained from Tocris Bioscience and administered i.p. immediately post-burn and twice daily (24 hr and 48 hr post-burn, respectively). Liposomal clodronate was purchased from Nico van Rooijen, PhD (Faculty of Medicine). They were prepared as previously described (Van Rooijen and Sanders, 1994). Mice were injected 48 hr prior to thermal injury and 48 hr post-thermal injury. Mice were sacrificed at 7 days post-thermal injury. Complete macrophages depletion was confirmed by immunostaining of Kupffer cells with CD14 (not shown).

Histology and Immunohistochemistry

Tissues collected were immediately fixed in formalin prior to paraffin embedding. Subsequently, tissues were sectioned and stained with H&E or incubated with UCP1 (Abcam) antibody followed by DAB staining. Image J (NIH) was used to determine adipocytes surface area. For immunofluorescence staining, Perilipin (Cell Signaling Technology) and VDAC1 (Abcam) were used according to recommended manufacturer's recommendations and mounted on DAPI-containing mounting medium (Vectashield). Imaging was performed on a LSM confocal microscope (Zeiss).

Transmission Electronic Microscopy

Fat tissues were fixed in universal fixative. The tissues were then dehydrated and infiltrated with Epon Araldite (E/A) resin. Following polymerization, tissue sections were counterstained and imaged using the Hitachi H7000 transmission electron microscope (Microscopy Imaging Laboratory, University of Toronto).

Western Blotting

Proteins from fat tissues were extracted and separated on SDS-PAGE. Western blotting was performed using COX IV, UCP1 antibody, VDAC1 (Abcam), and β-actin antibodies (Thermo Fisher Scientific). Proteins were visualized by enhanced chemiluminescence.

qPCR

RNA from eight patients per group was extracted with Trizol (Invitrogen). Specific primers yielding single specific amplicon were chosen, and qPCR was performed with sybr green Supermix (Bio-Rad).

Statistics

Unpaired Student's t test was used to determine significance. Significant results were established for $p < 0.05$ (*) and $p < 0.01$.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and four figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2015.10.028.

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