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Inverse distribution of uncoupling proteins expression and oxidative capacity in mature adipocytes and stromal-vascular fractions of rat white and brown adipose tissues

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Abstract To investigate relationships between the uncoupling protein (UCP) family and oxidative metabolism in fat pads, we measured the cytochrome oxidase activity, used as an index of oxidative capacity, and the mRNA content encoding UCP1, UCP2 and UCP3. Most oxidative potential was found in the stromal-vascular fraction (SVF) of brown fat and in mature adipocytes of white fat (inguinal and periovarian). Considering the whole fat pads, the oxidative potential observed in mature white adipocytes fraction was not negligible compared with that of brown adipocytes fraction. UCP1 and UCP3 were expressed exclusively in mature brown adipocytes. Whatever the deposit, UCP2 mRNA was mainly localized in the SVF. These results indicate that, in fat, high oxidative potential is not necessarily linked to high UCPs transcripts content and point out the oxidative capacity of SVF from brown fat.

Key words: Oxidative potential; Uncoupling protein; Adipose tissue; Cell fraction; Mitochondrion

1. Introduction

Brown (BAT) and white (WAT) adipose tissues play different roles in energy metabolism, being respectively an energy-dissipating and an energy-storing tissue.

The former, often located around large vessels, is involved in both non-shivering and diet-induced thermogenesis mainly occurring in hibernators, small and newborn mammals [1]. Within the tissue, brown adipocytes are specialized in heat production. They have a multilocular appearance and a high density of mitochondria [2,3] which are characterized by a high respiratory capacity, correlated with high content and activity of oxidative enzymes (succinate dehydrogenase, cytochrome oxidase (COX), etc.) [4]. The inner mitochondrial membrane also includes the specific uncoupling protein-1 (UCP1) which dissipates the proton gradient created by the respiratory chain. The energy transiently accumulated in the gradient is thus dissipated as heat instead of being used by ATP synthase. This uncoupling results in accelerated respiration and oxidative metabolism in brown adipocytes [5]. Intense COX activity is thus usually considered to be linked to the high thermogenic activity of BAT [6–9].

Contrary to BAT, the main function of WAT is to store energy as triglycerides in numerous and abundant deposits (retroperitoneal, periovarian, inguinal, etc.). Storage takes place in unilocular white adipocytes, which are classically described as having a fairly modest mitochondrial apparatus. However, some observations have pointed out the importance of mitochondria in WAT. Thus, expression of UCP1 from the aP2-gene promoter in WAT of transgenic mice results in increased energy expenditure [10], suggesting that the mitochondrial oxidative potential of white fat is quantitatively significant at the level of the whole organism. Furthermore, another UCP isoform expressed in a wide range of tissues, UCP2, has been cloned [11]. Like UCP1, UCP2 functions as a mitochondrial uncoupler when over-expressed in yeast [11,12], suggesting that it can increase oxidative metabolism. UCP2 is the only isoform of the UCP family expressed at a high level in white fat [11]. The presence of this UCP could thus participate in the oxidative metabolism of WAT and induce some energy dissipation by this tissue. Nevertheless, this putative role in energy expenditure is brought into question by the physiological regulation of the UCP2 gene observed in fasting experiments, a well-known condition of energy conservation in which UCP2 mRNA level is increased in human adipose tissue and rat skeletal muscle [13]. A third UCP, UCP3, which is significantly expressed in BAT and muscles, has also been identified [14,15]. It is noteworthy that the cell type responsible for its expression in adipose tissue is unknown.

The present study aimed to compare oxidative potential, assessed by COX activity, and UCP mRNA expression in BAT and WAT as well as in their respective cell fractions, in mature adipocytes versus stromal-vascular cells.

2. Materials and methods

2.1. Animals

Female Wistar rats (180–200 g) were obtained from a commercial stock-breeder (Iffa-Credo, France) and fed ad libitum (UAR A04). The animals were reared at 20°C under a constant photoperiod (12–12: L-D). They were killed by decapitation.

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Abbreviations: UCP, uncoupling protein; SVF, stromal-vascular fraction; IBAT, interscapular brown adipose tissue; POWAT, periovarian white adipose tissue; IWAT, inguinal white adipose tissue; COX, cytochrome oxidase

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2.2. Adipose cell isolation

Interscapular BAT, periovarian (POWAT) and inguinal (IWAT) white adipose tissues were carefully dissected to remove adhering tissues, capillaries and contaminating WAT surrounding the interscapular brown deposit. They were digested separately at 37°C with 0.5 mg collagenase type II (Sigma, St. Louis, MO, USA) per gram of tissue for 45 min (WAT) or with 15 mg collagenase type II per gram of tissue for 30 min (BAT), in oxygenated Krebs solution (pH 7.4) containing 20 mM HEPES, 3 mM glucose and 3.5% free fatty acid BSA, under maximal agitation for BAT and slow agitation for WAT. Tissue digests were filtered and floating mature adipocytes were separated from the subnatant, i.e. the stromal-vascular fraction (SVF), which contains all other adipose tissue cells. These cells were pelleted by centrifugation (20 min, 500×*g*, 4°C). Adipocytes and SVF were used separately for total RNA extractions or COX activity measurements.

2.3. Total RNA extraction and Northern blot analysis

Cell fraction total RNAs were prepared by the acidic guanidium isothiocyanate method [16] and adipose tissue total RNAs with Tripure Isolation reagent (Boehringer, Mannheim, Germany). 15 μg of total RNAs were size fractionated on denaturing formaldehyde (1.1%) agarose gel (1.2%), transferred onto a GeneScreen membrane (NEN, Boston, MA, USA) and UV cross-linked. After prehybridization (2 h at 42°C in phosphate buffer 0.1 M pH 6.5, formamide 45%, SSC 4X, SDS 0.1%, Denhardt 5X, salmon sperm DNA 75 μg/ml), the membrane was hybridized at 42°C for 16 h in the same buffer (but with Denhardt 1X) containing the radiolabeled probe. After hybridization, the membrane was washed in SDS 0.1%/SSC 2X–0.1X solutions between 42°C and 65°C and was exposed to a Kodak X-OMAT AR film at −80°C.

2.4. Probes

Approximately 50 ng of probe was ^{32}P-labeled by the random priming method (Mega prime DNA Labelling system, Amersham, Arlington Heights, IL, USA). Unincorporated dNTPs were removed using QIAquick Nucleotide Removal kit (QIAGEN, Chatsworth, CA, USA).

The different probes used were pBR 325 ST41 containing the total mouse mitochondrial genome [17], or obtained by PCR as rat UCP1 cDNA from positions 261 to 1086 of the coding phase, mouse UCP2 cDNA from positions 756 to 1234 of the coding phase, rat UCP3 cDNA from positions 676 to 1049 of the coding phase, and rat b-actin cDNA from positions 165 to 664 of the coding phase. All these probes were validated by sequencing after cloning.

2.5. COX activity measurement

Tissues were homogenized in 5 ml of cold (4°C) sucrose buffer (0.25 M, pH 7.2) containing 5 mM TES. After 2 washes in Krebs without BSA, adipocytes were homogenized in 3 volumes of sucrose buffer. Homogenates were centrifuged for 10 min at 10 000×*g* and 4°C. Lipids of each sample were discarded before rehomogenization. SVF pellets were homogenized in 400–800 μl of sucrose buffer. Protein concentrations were measured with a detergent-compatible protein assay based on the Lowry assay. Measurements of COX activity were carried out at 37°C by spectrophotometry at 550 nm in presence of reduced substrate cytochrome c (100 mM; Sigma, St. Louis, MO, USA), phosphate buffer (32 mM), sodium ascorbate (0.1 mM), aluminum chloride (0.4 mM) and lubrol (5 mg/ml for the brown SVF and 0.3 mg/ml for all other samples). These optimal concentrations of lubrol were determined for each type of sample. The cytochrome c was completely oxidized before ascorbate addition. According to the different specific activities of tissues or cell fractions, different amounts of proteins were used for measurements: 8 μg for BAT, 3 μg for brown adipocytes and SVF, 30 μg for WAT (tissues, adipocytes and SVF).

2.6. Statistical analysis

Northern blot autoradiograms were scanned with an SI densitometer (Molecular Dynamics, Sunnyvale, CA, USA) and signals were quantified by Imagequant software (Molecular Dynamics). Prism Software (GraphPad software, San Diego, CA, USA) was used for all statistical analysis of means by the paired *t*-test.

3. Results

3.1. Oxidative capacity of three different fat pads

The weight of interscapular brown adipose tissue (IBAT) was significantly lower ($P < 0.001$) than the weights of POWAT and IWAT: 0.38 ± 0.04 g, 1.81 ± 0.14 and 2.32 ± 0.11 g, respectively. Furthermore, there was a significant difference ($P < 0.01$) between POWAT and IWAT weights.

To estimate the oxidative potential of each tissue, COX activity was measured. This was expressed per μM/min/mg protein or per μM/min/pad to estimate the specific enzyme activity in a given biological lysate or the oxidative potential of a whole pad, respectively. In IBAT, COX was 10-fold more active than in POWAT and 12-fold more than in IWAT (Fig. 1A). When COX activity was considered in the whole pad, IBAT had a total oxidative potential 25-fold higher than POWAT and IWAT (Fig. 1B).

The mitochondrial transcript level was higher in IBAT than in POWAT and IWAT (Fig. 3A). After quantification, the profile was almost the same as specific COX activity (data not shown).

3.2. Oxidative capacity comparison between mature adipocytes and SVFs of three fat pads

Brown adipocytes are known to have a high oxidative potential and to be responsible for the thermogenic function of brown fat. So we compared their oxidative potential with that

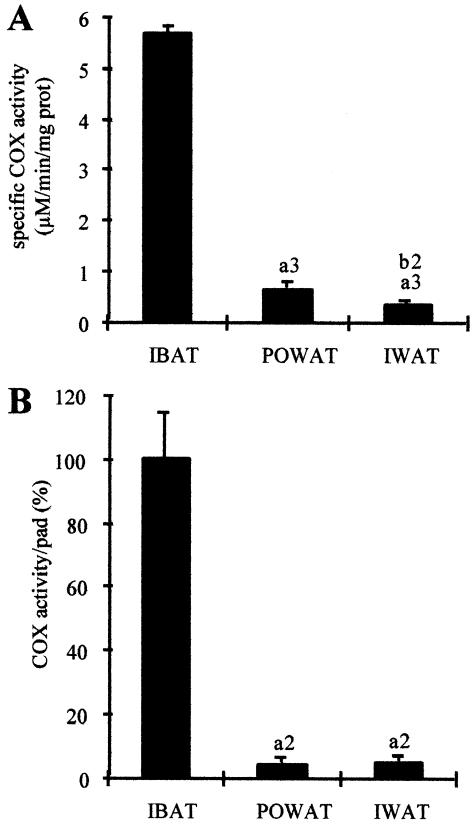

Fig. 1. COX activity in rat IBAT, IWAT and POWAT fat pads. COX activity is expressed per μM/min/mg of protein (A) or per μM/min/whole fat pad with values of IBAT fixed at 100% (B). Results express the mean ± S.E.M. of six independent values and statistical significance (paired *t*-test) is expressed as (a) versus IBAT; (b) versus POWAT; (2) $P < 0.01$; (3) $P < 0.001$.

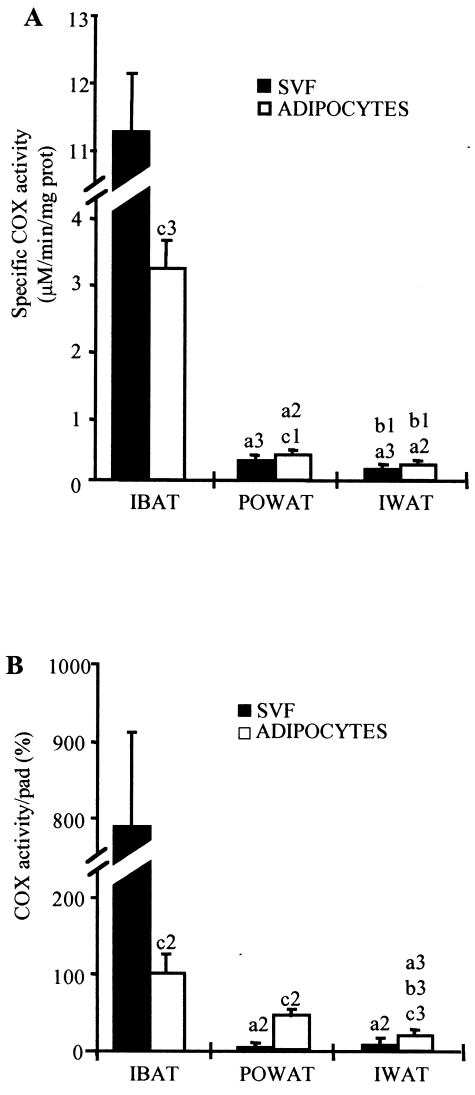

Fig. 2. COX activity in SVF and adipocyte fractions of rat IBAT, IWAT and POWAT fat pads. COX activity is expressed per μM/min/mg of protein (A) or per μM/min/whole fat pad with values of IBAT adipocytes fixed at 100% (B). Results express the mean ± S.E.M. of five independent experiments and statistical significance (paired *t*-test) is expressed as: (a) versus the same fraction of IBAT; (b) versus the same fraction of POWAT; (c) adipocyte versus respective SVF; (1) $P < 0.05$; (2) $P < 0.01$; (3) $P < .0.001$

of the SVF containing all the other cells of the tissue. The same comparison was carried out for adipocytes and SVF of POWAT and IWAT. Adipocytes and SVF were isolated from IBAT, POWAT and IWAT of the same rat and the COX activity was measured in each fraction. Absence of contamination between cell fractions was checked by using the aP2 probe which detects an mRNA exclusively expressed in mature adipocytes.

Specific COX activity in IBAT was 3.6-fold lower in adipocytes than in the SVF (Fig. 2A). In WAT, a different pattern was observed: COX activity in adipocytes was slightly higher than in the SVF, however, the difference was significant in POWAT and not in IWAT. In order to estimate the share of the oxidative potential of each cell population in a given pad, COX activity in brown adipocytes was set at 100%. Compared with the whole brown adipocyte fraction for a pad, brown SVF had a 8-fold higher oxidative potential. In other words, almost 90% of the oxidative potential of the whole pad was localized in the SVF deprived of UCP1 (Fig. 2B). Conversely, the POWAT mature adipocyte fraction had an oxidative potential 2-fold lower than that of brown adipocytes and represented 90% of the oxidative potential of the whole POWAT. The IWAT mature adipocyte fraction had an oxidative potential 5-fold lower than that of brown adipocytes and represented 60% of the oxidative potential of the whole IWAT (Fig. 2B). Furthermore, the differences of mitochondrial transcript levels between cell fractions (Fig. 3B and quantification not shown) were nearly the same as those observed for COX specific activity.

3.3. *UCP mRNA expression in three fat pads and their respective cell fractions*

As shown in Fig. 3A, UCP1 mRNA was strongly detected in IBAT, faintly in POWAT and was undetectable in IWAT. It was only present in brown adipocytes (Fig. 3B). UCP3 mRNAs were present only in IBAT and brown adipocytes (Fig. 3). UCP2 mRNA content was 3- and 4.5-fold higher in IWAT than in POWAT and in IBAT, respectively (Fig. 4A). Fig. 4B shows that the UCP2 transcript was found at a lower level in adipocytes than in SVF whatever the tissue examined (although the difference was not statistically significant in IWAT). The difference was higher in WAT than in IBAT.

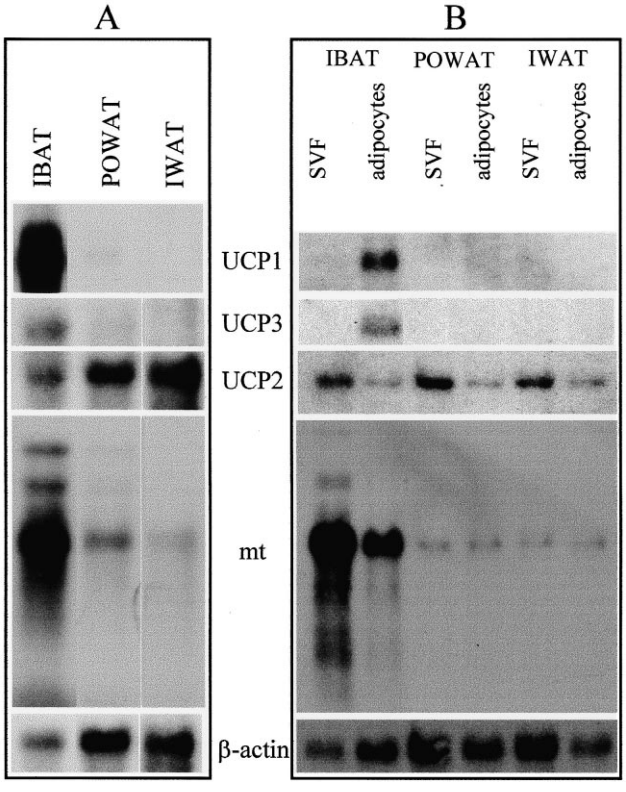

Fig. 3. Northern blot analysis of the mRNA content of UCPs and mitochondrial transcripts in rat brown and white fat pads and their respective cell fractions. Total RNA from one BAT, POWAT or IWAT (A) or from the respective SVF and adipocyte fractions isolated from four rats (B) were blotted and hybridized with probes for rat UCP1, mouse UCP3, rat UCP2, mouse mitochondrial (mt) genome and rat b-actin. This figure corresponds to representative Northern blots from one experiment.

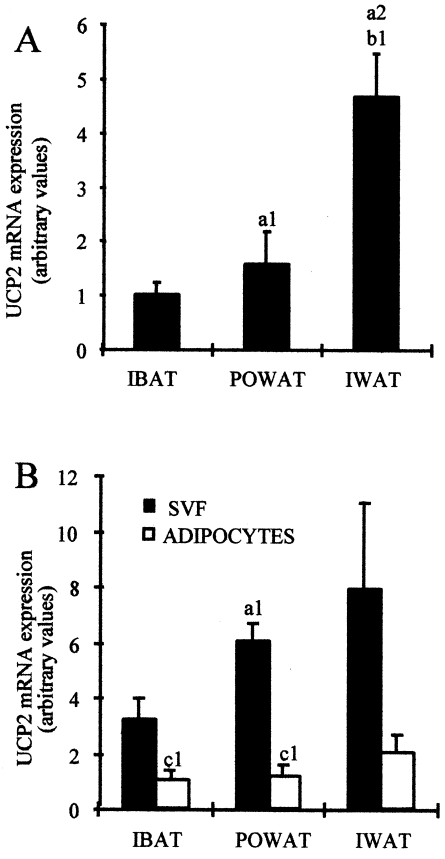

Fig. 4. UCP2 mRNA content in brown and white fat pads and their respective cell fractions. Northern blots were performed as described in Fig. 3, with total RNAs from BAT, POWAT and IWAT (A) or from their respective cell fractions (B), and were hybridized with the probe for rat UCP2. Results express the mean ± S.E.M. of three (adipocytes and SVF of POWAT) or six independent experiments. Arbitrary values of IBAT adipocytes were set at 1. Statistical significance (paired *t*-test) is expressed as: (a) versus IBAT, (b) versus POWAT, (c) adipocyte versus respective SVF; (1) $P < 0.05$; (2) $P < 0.01$.

4. Discussion

In the whole brown interscapular fat pad, we found a high level of COX activity, with values consistent with those expected. This high COX activity was associated with high levels of UCP1 and mitochondrial transcript contents (Figs. 2C and 3A). This strong BAT oxidative potential was expected to be mainly localized in thermogenic UCP1-containing brown adipocytes. Surprisingly, the brown SVF was responsible for the major part of the oxidative potential of the whole tissue. Any possible contamination of brown SVF with mature adipocytes was excluded by the absence of UCP1 mRNA detection on Northern blots. Among the different cell types contained in the SVF, brown preadipocytes (i.e. adipocytes still free from lipid) have been described as mitochondria-rich [9] and could thus be good candidates for the strong oxidative potential measured. However, an alternative explanation could be that the oxidative capacity per mitochondrion from mature brown adipocytes may be lower than that per mitochondrion from SVF cells. The association generally made, in BAT, between oxidative potential and thermogenic activity is thus erroneous. Measurement of COX activity or mitochondria transcripts rather reflects the oxidative potential of non-thermogenic cells within the tissue, whereas UCP1 mRNA level and UCP1 protein content are the best criteria for evaluating thermogenic capacity. This assertion is in agreement with the observation made by Kozak and coworkers in mice with targeted disruption of the UCP1 gene: although their IBAT still had a high mitochondria content, these animals were cold-intolerant [18]. Moreover, Kozak et al. demonstrated that UCP2 expression increases in their brown adipocytes. Such a location again raises questions about the physiological function of UCP2. It in fact occurred in the same location as UCP1 and was not sufficient to compensate the absence of UCP1 in UCP1 knockout mice with regard to cold sensitivity.

Typical WAT is known to be devoid of UCP1 and to contain few mitochondria [19,20], as confirmed in our study by Northern blot hybridization (Fig. 3A). These data are consistent with the rather low COX activity measured in WAT (Fig. 1A,B). Interestingly, specific enzyme activity was higher in POWAT than in IWAT. In the rat, it has been suggested that POWAT could contain brown preadipocytes, recruited in response to cold exposure [21]. The intermediate level of POWAT COX activity could thus reflect its heterogeneous composition of preadipose cells. Fig. 2A,B demonstrate that in WAT, for the whole pad, the oxidative potential was higher in mature adipocytes than in SVF. Conversely to brown adipocytes in IBAT, the white adipocytes of WAT are the major site of COX activity in the tissue. It follows that the total oxidative potential associated with white adipocyte fractions of POWAT and IWAT was not really different from the total oxidative potential provided by brown adipocytes in IBAT. This led us to consider that white adipocyte mitochondria and associated functions might be more important than was formerly believed. With respect to previous classical views, other observations provide further arguments in favor of such an idea. Transgenic mice expressing UCP1 in white adipocytes show increased energy expenditure [10] and reduced dietary obesity [22]. This effect was probably due to potentially active mitochondria already present in WAT. Furthermore, various microcalorimetric assays have measured heat production in white adipocyte cultures [23] and this thermogenesis has been found to be largely of oxidative origin and not insignificant [24].

UCP2 has been identified on the basis of sequence homologies with UCP1 and its uncoupling activity has been recently demonstrated [11,12]. The UCP2 gene is widely expressed and has been localized on a chromosomal locus linked to hyperinsulinemia and obesity. These data lead to the hypothesis that it could contribute to energy consumption. Whatever the adipose tissue examined, UCP2 mRNA was mainly localized in the SVF. Interestingly, the variations of UCP2 mRNA level observed between whole fat pads are only the consequence of differences between their respective SVFs. The UCP2 mRNA levels measured on whole fat pads or biopsies must thus be cautiously interpreted, keeping in mind the relative distribution of this transcript in the different adipose tissue fractions. During primary cultures of white or brown SVF, the UCP2 mRNA level has been found to increase progressively until white or brown adipocytes reach their full differentiation state [25,26]. Our results seem to be in contradiction with these previous findings, but it must be emphasized that the SVF is a very heterogeneous tissue fraction: it contains very different cell types, including preadipocytes but

also fibroblasts, endothelial cells, mast cells and monocytes/macrophages. A high level of UCP2 mRNA has been reported in macrophages [11,27]. This type of cell could thus be a good candidate for UCP2 mRNA expression, but other cell types are not excluded. It is often assumed that UCP activity must be associated with high COX activity. At least at the mRNA level, this general idea was confirmed on whole IBAT (with UCP1) in the present study but was proved incorrect on whole WAT (with UCP2) and on adipose tissue cell fractions. Just as UCP1 mRNA localization in BAT was dissociated from COX activity, UCP2 in WAT was also localized in the lowest oxidative fraction, i.e. the SVF. It is striking that the brown SVF shows both a strong UCP2 level and a high COX activity. These data imply that the brown SVF may have a different cell type composition from that of white SVF and/or that the cell type with a high oxidative potential may be different from that which expresses UCP2.

To sum up, the present study draws attention to the surprisingly high oxidative potential of brown SVF and the not insignificant potential of white adipocytes. Furthermore, maximal COX activity and UCPs transcripts were inversely distributed in adipose tissue fractions. Our results emphasize the physiological relevance of non-adipose cells in adipose tissue. They are the site of the main expression of UCP2, and where BAT is concerned, of the major part of the oxidative potential.

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