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Combination of two oxidant stressors suppresses the oxidative stress and enhances the heat shock protein 27 response in healthy humans

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Abstract

We tested the hypothesis that the combination of 2 oxidant stressors (hyperoxia and fatiguing exercise) might reduce or suppress the oxidative stress. We concomitantly measured the plasma concentration of heat shock proteins (Hsp) that protect the cells against the deleterious effects of reactive oxygen species. Healthy humans breathed pure oxygen under normobaric condition for 50-minute periods during which they stayed at rest or executed maximal static handgrip sustained until exhaustion. They also repeated handgrip bouts in normoxic condition. We performed venous blood measurements of 2 markers of the oxidative stress (thiobarbituric acid reactive substances and reduced ascorbic acid) and Hsp27. Under normoxic condition, the handgrip elicited an oxidative stress and a modest increase in plasma Hsp27 level (+7.1 ± 5.4 ng/mL). Under hyperoxic condition, (1) at rest, compared with the same time schedule in normoxic condition, we measured an oxidative stress (increased thiobarbituric acid reactive substances and decreased reduced ascorbic acid levels) and the plasma Hsp27 level increased (maximal variation, +12.5 ± 6.0 ng/mL); and (2) after the handgrip, the oxidative stress rapidly disappeared. The combination of both hyperoxia and handgrip bout doubled the Hsp27 response (maximal variation, +24.8 ± 9.2 ng/mL). Thus, the combination of 2 hits eliciting an oxidative stress seems to induce an adaptive Hsp27 response that might counterbalance an excessive production of reactive oxygen species.

1. Introduction

In humans working under normoxic condition, static handgrip sustained until exhaustion increases the blood levels of oxidative stress markers [1]. On the other hand, normobaric hyperoxia produced by pure oxygen breathing also elicits an oxidative stress in culture cells [2], different rat tissues [3], and the human blood [4,5].

We already showed that the combination of 2 oxidant stressors (hypoxia and handgrip exercise or fatiguing electrical muscle stimulation) markedly attenuated or even suppressed the oxidative stress assessed in the human blood [1,6] and rat muscles [6]. On the other hand, reoxygenation of chronic hypoxemic patients to suppress the baseline hypoxemia restored the postexercise oxidative stress [7]. Thus, under hypoxemic condition, a second hit represented by a fatiguing muscle contraction seems to exacerbate the defense against the oxidative stress.

Numerous data suggest that the heat shock proteins (Hsp) protect the cells against the deleterious effects of reactive oxygen species (ROS). Expression of Hsp72 [8], Hsp25 in rats [9], and Hsp27 in humans [10] reduces the oxidative stress. Heat shock protein 70 and Hsp72 reduce the oxidative stress via the activation of antioxidants [8], whereas Hsp25 and Hsp27 seem to either directly scavenge the free radicals [9] and/or protect against the toxicity of ROS on constitutive cell proteins but not against their production [10]. Heat shock protein 20, Hsp25, Hsp27, and Hsp70 formation occurs in

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Institutional approval: the research was approved by our institutional ethics committee, and each subject gave an informed consent.

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contracting muscles [8,9,11]. Human studies clearly indicate that the small Hsp (Hsp20 and Hsp27) rapidly appear in muscle biopsies after a maximal eccentric exercise [12] and also in the venous blood after a maximal cycling exercise [13]. By contrast, the postexercise increase in Hsp70 level in muscle tissue and venous blood was markedly delayed [12,13] or absent [14]. Increased Hsp expression may be triggered by various cell stresses, including excessive heat, hypoxia, and muscle contraction at a high strength [14]. Hyperoxia was demonstrated to increase the expression of Hsp70 messenger RNA in human airway epithelial cells studied in vitro and in vivo [15,16]. Furthermore, Hsp70 attenuates the lipid peroxidation in the human airway epithelial cell line exposed to hyperoxia [17]. These studies were motivated by the frequent use of inhalation of enriched oxygen gas mixture in anesthesia and resuscitation and also for the treatment of severe respiratory insufficiency. On the other hand, we found no data in the literature on hyperoxia as a potential inductor of Hsp in resting or contracting muscle, despite the numerous observations that training in normobaric hyperoxia often increases the muscle performance [18-20].

Based on our previous observations in hypoxemic subjects [1,6,7], we hypothesized that the addition of 2 hits (hyperoxia plus fatiguing exercise) might suppress the oxidative stress elicited by the first hit. This effect might result from an increased Hsp release. In the present study, the subjects were asked to perform a maximal handgrip exercise sustained until exhaustion. We measured 2 blood markers of the oxidative stress (thiobarbituric acid reactive substances [TBARS] and reduced ascorbic acid [RAA]) and the plasma Hsp27 level. Measurements were performed in separate sessions where the subjects breathed air or pure oxygen at rest or executed the handgrip bouts in normoxia (single hit) or hyperoxia (2 hits).

2. Materials and methods

2.1. Subjects

Eight healthy white subjects (3 women, 5 men; mean age, 32 ± 6 years; mean weight, 70 ± 4 kg) participated in the study. None were involved in an exercise training program. Written informed consent was obtained from all subjects, and the protocol was approved by our Institutional Ethics Committee.

2.2. Protocols

The subjects performed the 4 tests in a random order: (1) at rest, room air inhalation (normoxia) for a 50-minute period; (2) also at rest, pure oxygen breathing (normobaric hyperoxia) for a 50-minute period; (3) handgrip tests in normoxia; and (4) handgrip tests in normobaric hyperoxia. One week elapsed between successive sessions. In all conditions, the subjects breathed through a face mask. To produce normobaric hyperoxia, the subjects breathed pure oxygen stored in a 100-L Douglas bag and administrated through a 2-way valve (Hans Rudolph, Kansas City, MO) connected to the face mask. Arterial blood gases, including arterial partial pressures of oxygen and carbon dioxide and arterial pH, were analyzed in 100 μL arterialized blood sampled from the ear lobes (Corning-Chiron model 860; Bayer, East Walpole, MA) when subjects breathed room air (pre- and postexposure to hyperoxia) and at the 20th and 50th minute of hyperoxia in both resting and exercising conditions. The percutaneous oxygen saturation was continuously measured throughout the exercise challenge and the recovery period using an infrared analyzer (Nellcor model N3000, Covidien-Nellcor, Boulder, CO). Fig. 1 shows a schematic representation of the protocols and also the timing of venous and arterialized blood samples for the different analyses.

2.3. Force measurements

The subjects were seated comfortably. One forearm was maintained in a horizontal prone position in an anatomical device specially built for the experiment that allowed isometric handgrip [1]. The subjects were given visual

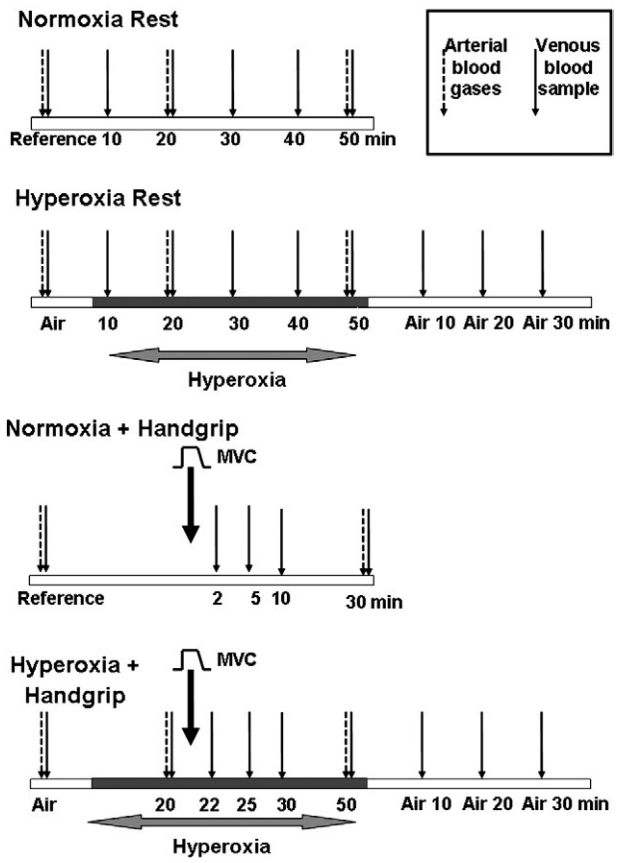

Fig. 1. The different protocols undergone by each volunteer. Arrows indicate the epochs of ear capillary blood sampling to measure the arterial blood gases and venous blood sampling to dose the markers of oxidative stress and Hsp27.

feedback from a load cell (ZC Scaime, Annemasse, France) to keep the preset force level constant, that is, at the maximal voluntary contraction (MVC). The force signal was continuously addressed to a chart recorder (TA 4000; Gould, Ballainvilliers, France). At the beginning of the experiment, the subject was instructed to perform 3 MVCs sustained for 3 seconds, with a 1-minute interval between each MVC. The highest force recording of the 3 contractions was considered as the MVC. Muscle fatigue was induced by a sustained maximal handgrip trial. The subjects were asked to breathe normally during the handgrip exercise. In sustained handgrip trials, the endurance time to fatigue was measured from the onset of plateau contraction to the first significant fall in force, that is, a 20% fall of the hold force. The contraction was interrupted in both fatigue tests when the force fell to 50% of its initial value.

2.4. Biochemical analyses

All biochemical analyses were treated in our laboratory. A catheter (Neofly 21G, Viggo-Spectramed; Johnson & Johnson, Brussels, Belgium) was inserted in an antecubital vein. Six milliliters of heparinized blood was sampled at different epochs of the protocol. Blood lactic acid concentration was measured with an enzymatic electrode (Corning-Chiron model 860, Bayer). Plasma TBARS and RAA were analyzed according to procedures already published elsewhere [1,5-7,21,22] and based on the original methods by Uchiyama and Mihara [23] for TBARS and Maickel [24] for RAA. Plasma Hsp27 level was measured with high-sensitive enzyme-linked immunosorbent assay kits (Human Hsp27 Total; BioSource International, Camarillo, CA; supplied by Invitrogen, Eragny sur Oise, France). The limit of detection of Hsp27 assays was less than 0.3 ng/mL. All measurements were made in duplicate, and the coefficient of variation was inferior to 5%. In resting normoxic subjects, all Hsp27 levels were higher than the detection limit.

In all experimental conditions, the subjects first breathed room air when they stayed at rest. The first venous blood sample was performed 20 minutes after placement of all measurement apparatus. In sessions where the subjects continued to breathe room air, further venous blood samples were performed at the 10th, 20th, 30th, and 50th minute. When subjects stayed at rest and subsequently breathed pure oxygen, venous blood samples were performed at the end of each 10-minute epoch during the 50-minute period of hyperoxia, then at the end of each 10-minute epoch during the posthyperoxic recovery period (Fig. 1).

In sessions where the handgrip bouts were executed in normoxic condition, venous blood sampling was performed at rest; within the 2 first-minute periods after the handgrip; and then at 5, 10, and 30 minutes after the static exercise had stopped (Fig. 1). When a fatiguing handgrip was executed during hyperoxia, venous blood sampling was performed at rest in air condition and at the 20th minute of hyperoxia, before beginning the handgrip; then samples were repeated within the 2 first-minute periods after the handgrip and at the 27th, 30th, and 50th minute of hyperoxia. Blood sampling was also repeated 10 and 30 minutes after hyperoxia had stopped (Fig. 1).

2.5. Statistical analyses

Data are presented as mean ± SEM. The normal distribution of variables was verified with the Kolmogorov-Smirnov test. For temporally repeated data, the changes over time were determined using analysis of variance for repeated measures when variables were normally distributed or a Friedman test for repeated measures when they were not. Time differences were identified using Tukey multiple comparison test. Spearman regression analysis was used to correlate the corresponding values of TBARS and Hsp27 measurements in the same subjects. Significance was set at the .05 level.

3. Results

3.1 Resting condition

As shown in Fig. 2, pure oxygen breathing elicited marked hyperoxia, the oxygen partial pressure in arterial blood rising to 395 ± 15 mm Hg between the 20th and 50th minute of hyperoxia. No hypercapnia occurred. We ensured that high levels of hyperoxia were maintained in all experimental conditions, including those necessitating execution of a handgrip exercise. By contrast, in resting individuals, the oxygen partial pressure in venous blood was not significantly elevated (control, 41 ± 5 mm Hg; hyperoxia 20th minute, 47 ± 11 mm Hg; hyperoxia 50th minute, 52 ± 9 mm Hg). Thus, the arteriovenous oxygen difference considerably increased under hyperoxic condition; and this authorizes to suppose that the oxygen extraction was elevated in the same proportion. We measured a progressive decline in venous lactic acid level that became significant at the end of the 50-minute period of hyperoxia and persisted during the first 20 minutes of return to normoxia (Fig. 3).

In resting subjects breathing room air, the baseline plasma levels of TBARS, RAA, and Hsp27 slightly but not significantly differed between the successive sessions (Figs. 4, 6, and 7). Their baseline values stayed in the range of those measured in previous studies [1,5-7,13,21,22]. Fig. 4 superimposes the data obtained in the same resting individuals in 2 separate sessions (room air or pure oxygen breathing). Compared with data obtained with the same time schedule in normoxia, hyperoxia progressively increased the TBARS concentration throughout the 50-minute period of pure oxygen breathing (maximal TBARS variation, +0.71 ± 0.18 nmol/mL) and also reduced the RAA level at the 10th, 20th, and 30th minutes of hyperoxia (Fig. 4). Thus, normobaric hyperoxia really elicited an oxidative stress. The Hsp27 level significantly increased at the 10th minute of hyperoxia, and the changes were stable during all the

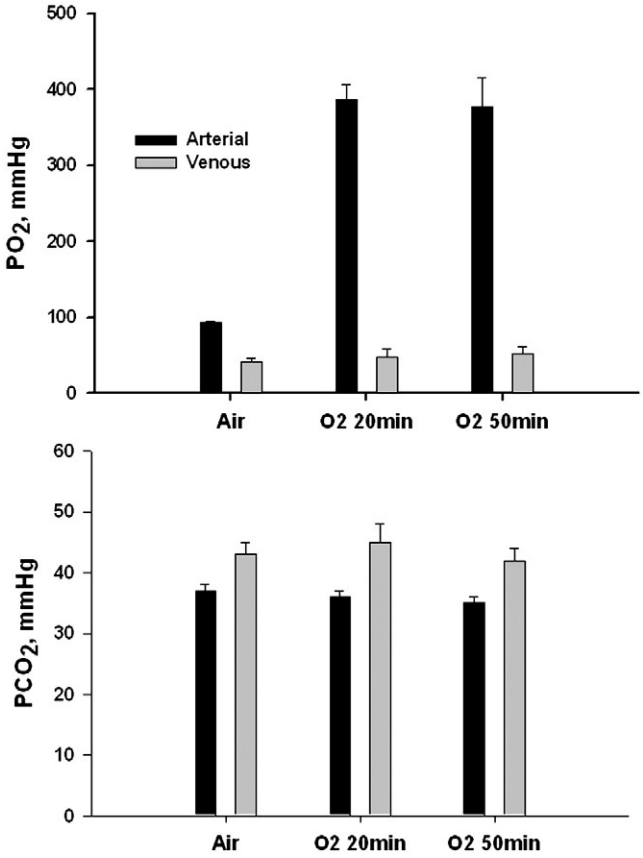

Fig. 2. Partial pressures of oxygen and carbon dioxide measured in arterialized and venous blood during air and pure oxygen breathing in resting subjects and in sessions where they executed a handgrip exercise. Values are mean ± SEM.

hyperoxic session (20th minute, +12.5 ± 6.0 ng/mL; 50th minute, +11.0 ± 13.0 ng/mL). The Hsp27 variations declined but persisted during the 30-minute posthyperoxic period. A

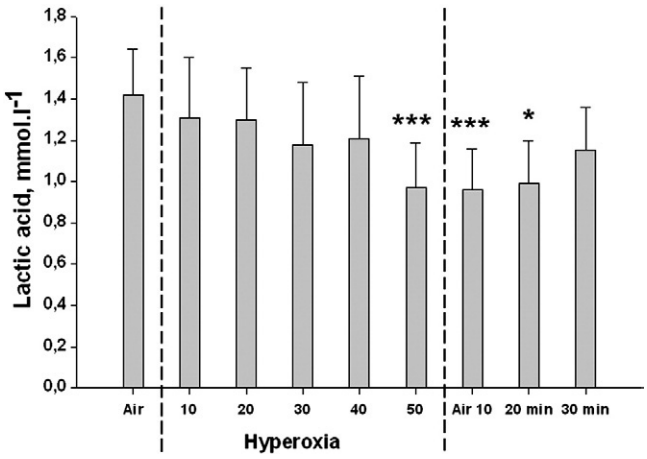

Fig. 3. Lactic acid concentration measured in venous blood at different epochs of the protocol in resting subjects. Values are mean ± SEM. Asterisks indicate significant reduction of lactic acid level compared with control (initial room air breathing period) (*$P < .05$, ***$P < .001$).

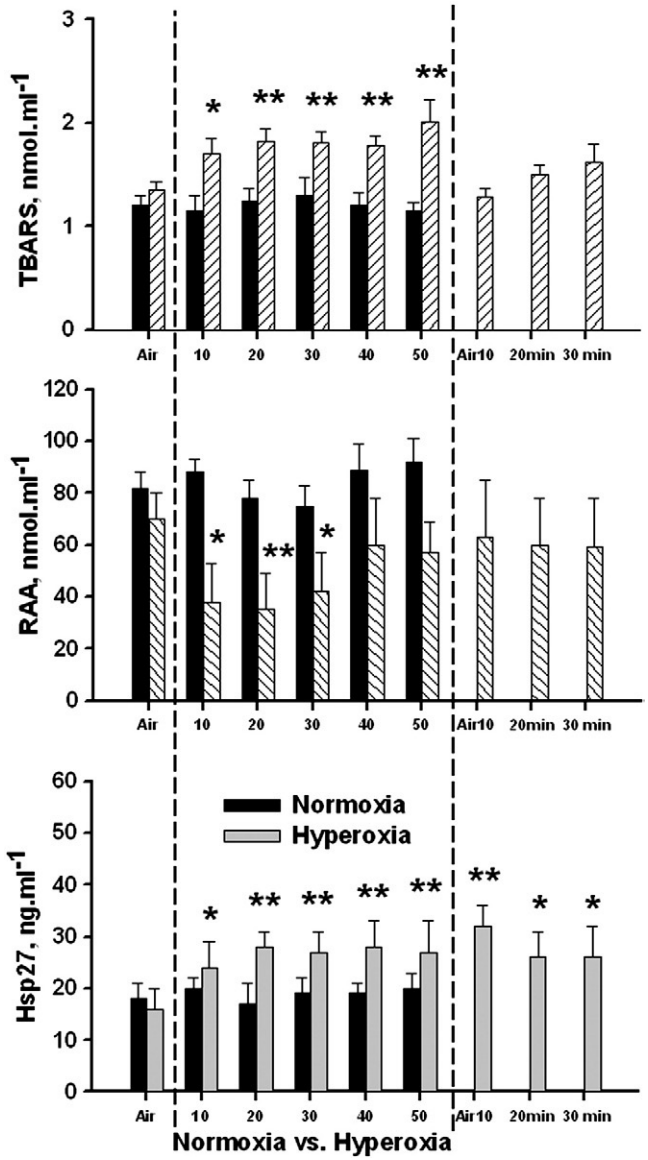

Fig. 4. Resting normoxic and hyperoxic conditions. Plasma levels of TBARS, RAA, and Hsp27 were measured when subjects continued to breathe room air (normoxia, black vertical bars) or pure oxygen for a 50-minute period (hyperoxia, dashed and gray vertical bars). Values are mean ± SEM. Asterisks denote significant changes compared with the corresponding initial air breathing period at rest (*$P < .05$, **$P < .01$).

significant positive correlation was found between Hsp27 and TBARS levels measured in normoxic then hyperoxic resting subjects (Fig. 5). Only Hsp27 and TBARS values measured at the 20th minute of hyperoxia were considered because in all our experimental hyperoxic conditions (including or not a handgrip bout), the subjects stayed at rest during this period while they breathed pure oxygen.

3.2 Handgrip test

The total duration of a sustained handgrip trial did not significantly differ between normoxia (64 ± 5 seconds;

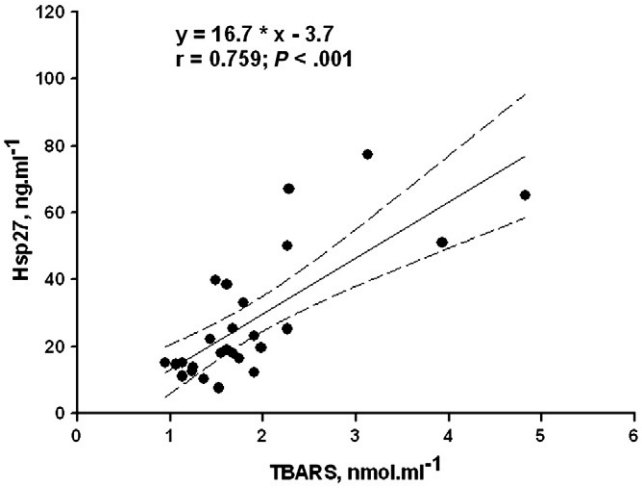

Fig. 5. Relationship between plasma levels of Hsp27 and TBARS measured in resting subjects breathing ambient air then pure oxygen for a 20-minute period. Spearman regression equation and regression line with ±95% confidence intervals are shown.

extreme values, 54-102 seconds) and hyperoxia (75 ± 7 seconds; extreme values, 47-100 seconds).

In normoxic condition, the handgrip exercise elicited significant changes in TBARS and RAA levels, that is, an oxidative stress, and also a modest but significant increase in plasma Hsp27 level (ΔHsp27 = +7.1 ± 5.4 ng/mL, $P < .05$) (Fig. 6).

When fatiguing handgrip bouts were executed in hyperoxic condition (Fig. 7), the initial increase in TBARS level measured at the 20th minute in resting hyperoxic condition persisted 2 minutes after the handgrip had stopped. Afterward, there was no further exercise-induced TBARS increase; and despite hyperoxia being continued, the TBARS level rapidly decreased, reaching the values measured in resting air condition. We also noted that the initial RAA decrease measured in resting hyperoxic condition was abolished by the handgrip bout. Thus, after the handgrip, RAA levels did not significantly differ from those measured in resting air condition. In the situation combining hyperoxia plus handgrip, the Hsp27 increase was markedly accentuated (at the 50th minute of hyperoxia and compared with data measured in air condition: ΔHsp27 = +24.8 ± 9.2 ng/mL, $P < .01$). The increase in Hsp27 level continued to be significant 30 minutes after hyperoxia had ended (ΔHsp27 = +11.1 ± 8 ng/mL, $P < .05$). Comparison of the sole handgrip-induced changes in biochemicals in normoxia and hyperoxia allows to highlight the differences between the 2 situations. The absolute values of handgrip-induced TBARS, RAA, and Hsp27 variations calculated between pre- and posthandgrip (30th minute) epochs were reported in Figs. 6 and 7. This clearly shows that hyperoxia inversed the TBARS response to the handgrip, but did not significantly modify the posthandgrip changes in RAA and Hsp27 levels. Thus, hyperoxia or handgrip alone elicited nearly the same Hsp27 increases (+11.0 ± 13.0 and +13 ± 9 ng/mL, respectively), whereas the combination of the 2 hits doubled the Hsp27 response.

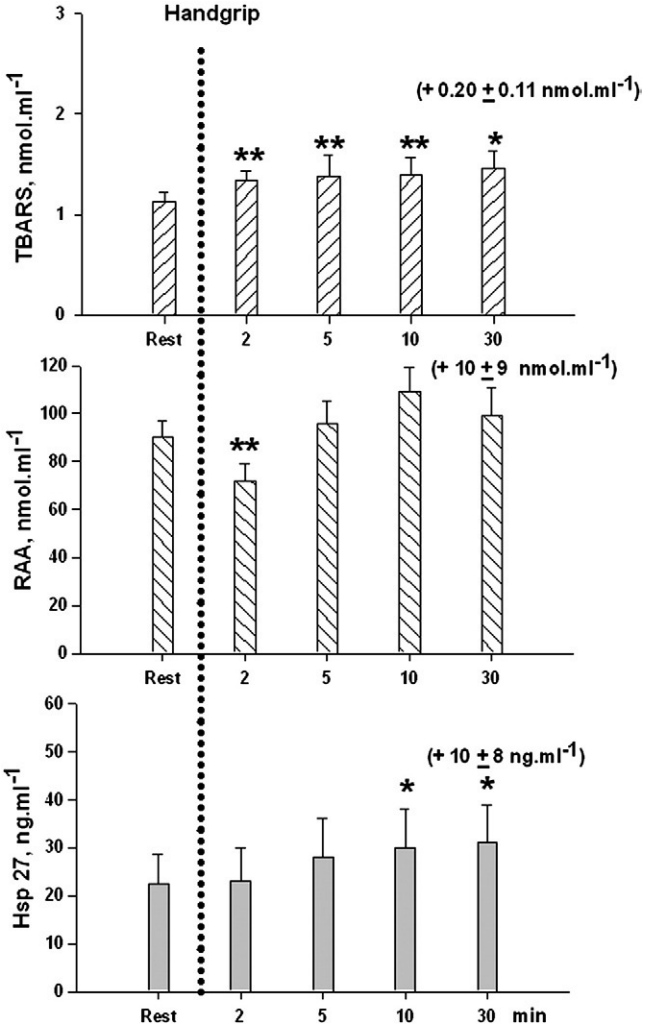

Fig. 6. Handgrip exercise in normoxic condition. Plasma levels of TBARS, RAA, and Hsp27 in resting subjects and then after they had executed a fatiguing handgrip exercise. Absolute mean values of the changes in each variable at the 30th minute after the handgrip bout are reported in parentheses. Values are mean ± SEM. Asterisks denote significant changes compared with control (initial air breathing period at rest) (*$P < .05$, **$P < .01$).

4. Discussion

The present study confirms that normobaric hyperoxia elicits an oxidative stress in resting subjects and also shows an associated increase in plasma Hsp27 level. Static handgrip exercise in normoxic condition also produces an oxidative stress and increases the Hsp27 level. By contrast, when the static handgrip was superimposed to hyperoxia, the initial oxidative stress initiated by hyperoxia no longer occurred. The combination of hyperoxia plus handgrip exercise elicited a marked increase in the plasma Hsp27 level. Because we already

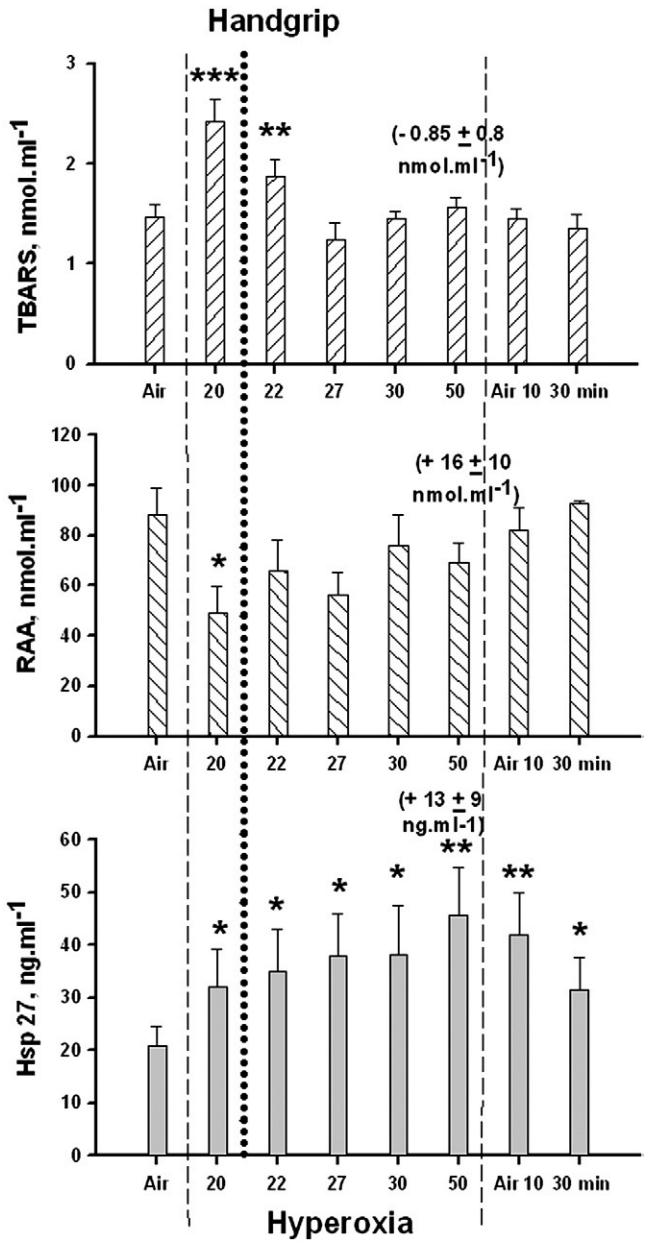

Fig. 7. Handgrip exercise superimposed to hyperoxia. Plasma levels of TBARS, RAA, and Hsp27 measured at rest in normoxia (air) then hyperoxia, after a handgrip bout executed when subjects continued to breathe pure oxygen, and during the 30-minute recovery period after hyperoxia (air 10 and air 30 minutes). Absolute mean values of the changes in each variable at the 30th minute after the handgrip bout are reported in parentheses. Values are mean ± SEM. Asterisks denote significant changes compared with control (rest) (*$P < .05$, **$P < .01$, ***$P < .001$).

showed that fatiguing static exercise under acute [1] and chronic [6,7] hypoxic condition did not produce an oxidative stress, it seems that the combination of 2 oxidant stressors (dysoxia plus exercise) promotes an adaptive response protecting the muscle against an excessive ROS production. The present study suggests that an enhanced release of stored Hsp27 might play a key role in this protective mechanism.

We found a highly significant positive correlation between plasma TBARS and Hsp27 levels measured at rest in normoxic and hyperoxic conditions. However, such correlation cannot help to assess a cause-to-effect relationship between the hyperoxia-induced ROS production and the elevated Hsp27 levels. We found one study in human lung cell-line [25] showing that an overexpression of Hsp27, Hsp72, and Hsp90 occurred after exposure to cadmium chloride, a major ROS inductor, and that this overexpression no longer occurred after treatment of cells with *N*-acetyl-L-cysteine that traps the free radicals. Thus, it seems that ROS are really able to trigger the Hsp27 production. Concerning the origin of elevated plasma Hsp27 levels in resting hyperoxic subjects, a rat study by Ahotupa and coworkers [3] has already shown that the ROS production markedly increases in lungs, brain, liver, and kidneys during exposure to normobaric hyperoxia. The observations by Ahotupa et al [3] authorize to speculate that an increased Hsp27 production could accompany the hyperoxia-induced oxidative stress in these target organs in our subjects. Besides, we may suppose that the rapid elevation of plasma Hsp27 level after handgrip bouts executed in normoxic or hyperoxic conditions results from the release of stored Hsp by the exercising muscle. Such a rapid increase in plasma Hsp27 concentration after a maximal cycling exercise was already reported by our team in healthy subjects [13].

The arteriovenous difference in PO_2, an indicator of the cellular respiration, was rarely measured under hyperoxic condition; and we only found one publication by Korkusho and Ivanov [26] who reported a marked increase in the arteriovenous oxygen difference in humans exposed to normobaric hyperoxia. This suggests that normobaric hyperoxia markedly increases the oxygen uptake. We also confirmed the reduction of the anaerobic glycolysis under hyperoxia, an effect already reported in the brain [27]. Such an increase in aerobic metabolism under hyperoxic condition might explain the elevated ROS production that is proportional to the mitochondrial activity [28]. Under normobaric condition, the hyperoxia-induced increase in ROS production was already demonstrated in the human blood [4,5].

In our study, the subjects performed the different protocols on separate weeks. This might explain some quantitative differences in the changes in oxidant-antioxidant status in response to hyperoxia. Indeed, when data in Figs. 4 and 7 are compared, the hyperoxia-induced TBARS increase measured at the 20th minute was significantly ($P < .05$) higher in Fig. 7 than in Fig. 4 (ΔTBARS = +0.92 ± 0.33 vs +0.58 ± 0.29 nmol/mL). We did not find any data in the literature on the reproducibility of hyperoxia-induced oxidative stress in the same individuals in normobaric or hyperbaric condition. Based on our previous observations of the variability of resting levels of plasma markers of the oxidant-antioxidant status in healthy subjects [1,5-7,13,21,22], we hypothesized that the stress-induced changes in these biomarkers might also differ when studied on separate weeks. It merits to be

underlined that the hyperoxia-induced increase in Hsp27 level did not significantly differ between the 2 sessions (ΔHsp27 = +11 ± 4 and +12 ± 6 ng/mL at the 20th minute of hyperoxia, respectively). Thus, the adaptive Hsp response to hyperoxia seems to be more reproducible than the oxidative one.

As reported above, we only found data in the literature on the effects of hyperoxia on Hsp70 expression in bronchial epithelial cells [15-17]; but similar effects on Hsp27 are not reported. It is well documented that exercise performed under normoxic condition induces an expression of the Hsp and addresses both the small (Hsp25-27) and large Hsp (Hsp70-72) in muscle fibers [8,9,11,12,14]. The elevated blood Hsp27 level after exercise might come from the leukocytes [29] and also from the contracting muscles. Indeed, in contrast with the large Hsp72 that difficultly crosses the myocyte membrane during and after exercise [14], significant increase in blood concentration of small Hsp27 was measured immediately after an exercise bout in humans [13].

We noted a clear dissociation between the effects of enhanced Hsp expression on TBARS and RAA. Indeed, in our study, the TBARS level went up under hyperoxia; but the plasma RAA level did not significantly vary. This agrees with the observations that Hsp27 did not increase the production of intracellular antioxidants (glutathione peroxidase, glutathione reductase, Mn superoxide dismutase) in muscle cells but might directly scavenge the free radicals [9] or oppose the ROS toxicity on constitutive proteins [10], reducing the lipid peroxidation measured by TBARS.

A major observation in the present study was the rapid increase in blood Hsp27 level after a short-duration handgrip executed in normoxic or hyperoxic condition. A handgrip bout only concerns the flexor digitorum muscles and thus a muscle mass markedly less than that solicited by a maximal knee extension [12] or a maximal cycling exercise [13], 2 situations where a significant increase in plasma Hsp27 level already occurred during the challenge and progressed thereafter. Thus, the main question is this: what are the stress-sensing and -transducing mechanisms that rapidly trigger the plasma Hsp27 release, even when a small muscle group contracts? Indeed, it is easy to understand that a general dysoxic (hypoxic or hyperoxic) condition elicits an excessive ROS production in numerous different tissues including the muscle and a general adaptive Hsp response. On the other hand, it is more difficult to understand how the local oxidative stress induced by the contraction of a small muscle may elicit an increase in plasma Hsp27 level. It is tempting to speculate that the nerve signals arising from active muscle might trigger the cell defenses against an excessive ROS production not only in the contracting muscle but also in other tissues. Recent observations indicate that Hsp27 [30] and Hsp90 [31] phosphorylation is related to the acetylcholine release in the pulmonary arteries and the colon. Acetylcholine-mediated Hsp phosphorylation is not demonstrated in skeletal muscle, but it is tempting to speculate that the motor cholinergic reflex response to the activation of muscle afferents [32] may participate in the Hsp activation. It must be underlined that the group IV metabosensitive muscle afferents are sensitive to both muscle hypoxia and hyperoxia [33,34]. Thus, the activation of the group IV muscle afferents may indirectly participate in the production and/or release of Hsp27 from the exercised muscle fibers. The possible involvement of nerve signals to induce Hsp activation merits exploration.

In conclusion, the present observations confirm our initial hypothesis that the addition of a second stressor attenuates or even suppresses the initial excessive ROS production elicited by a first oxidant stressor. We may only speculate on the intervention of Hsp27 in this particularly efficient protective response.

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