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Effect of Variable Glutathione Peroxidase Activity on H_2O_2-Related Cytotoxicity in Cultured Aortic Endothelial Cells (42150)

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Abstract. Primary cultures of porcine aortic endothelial cells were used to assess the effects of O_2 intermediates produced by 10–40 mU/ml xanthine oxidase (XO; +2 m*M* hypoxanthine) or 25–100 mU/ml glucose oxidase (GO; +5 m*M* glucose). A 60-min incubation in the presence of the enzyme systems resulted in a dose-dependent toxic effect with evidence of cytolysis (increased LDH release) and cell loss (decrease in DNA and protein content), when these indexes were measured 24 hr after completion of the enzyme reaction. Decreased [^{3}H]thymidine incorporation into DNA was the most sensitive index of cell dysfunction for both enzyme systems. The effects of various scavengers and enzymes indicated that H_2O_2 was the main O_2 intermediate involved in the cytotoxicity resulting from the XO–hypoxanthine reaction. Increased glutathione peroxidase activity associated with the addition of $2 \cdot 10^{-7}$ *M* selenomethionine to culture medium had a partial protective effect which could be related to an increased rate of H_2O_2 degradation. Evidence for increased DNA synthesis after injury was found in cells previously exposed to XO–hypoxanthine, the degree of increase in [^{3}H]thymidine incorporation being dependent on the intensity of the initial cytotoxicity. Cultured endothelial cells provide a useful tool to evaluate the role of O_2 intermediates in endothelial cell injury, to test the effects of protective agents, and to study the repair process.

Oxygen metabolites can be produced intracellularly when cells are exposed to high O_2 concentration or to the effects of certain chemical compounds (1) or released into the extracellular phase by activated neutrophils and monocytes (2). The interest in their biological action has stimulated the *in vitro* study of the effects of enzyme reaction capable of generating O_2 intermediates. Thus, a number of investigators have used systems involving xanthine oxidase (XO) and various substrates, such as xanthine, hypoxanthine, or acetaldehyde, with the production of O_2^-, H_2O_2, $OH^\cdot$, and $^1O_2$ (3–5). Others have studied the effect of H_2O_2 when formed under the action of glucose oxidase (GO).

Because endothelial cells (EC) appear to be especially vulnerable to the effects of O_2 radicals resulting from exposure to high O_2 concentrations, drugs and O_2^- metabolites released by polymorphonuclear leucocytes (PMN) (6–12), we have decided to study in greater detail their response to various doses of XO and GO, as well as the time course of the effects of O_2 metabolites so produced. Since the addition of selenomethionine (Se-Met) to culture is associated with an increase in glutathione peroxidase activity (11), we have also tested for a possible protective effect resulting from increased glutathione peroxidase activity. The data obtained confirm that H_2O_2, but not O_2^-, is involved in the cytotoxic effect following the enzyme reaction. Its action depends on both the duration and the intensity of its production. The protective effect associated with an increased glutathione peroxidase activity appears to be related to an increased rate of degradation of the H_2O_2 formed and not necessarily to lipid peroxide detoxification.

Material and Methods. The chemicals were of analytical grade and were generally purchased from Fluka AG (Buchs, Switzerland) or Merck (Darmstadt, W. Germany). *N*-Carbobenzoxy–Phe–His–Leu used for the measurement of angiotensin converting enzyme activity was obtained from Serva (Heidelberg, W. Germany). Superoxide dismutase (SOD; 2500–2700 U/mg from bovine red cells), horse heart cytochrome *c* type III, milk xanthine oxidase, reduced glutathione, glutathi-

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one reductase type III, NADPH tetrasodium salt type III, β-NADH, catalase (11.000–14.000 U/mg), scopoletin, horseradish peroxidase (150 U/mg), glucose oxidase (15.000 U/g), and hypoxanthine were obtained from Sigma Chemical Company (St. Louis, Mo.). [^{3}H]Thymidine ([^{3}H]TdR; 25 to 46 Ci/mmole) was bought from Radiochemical Center (Amersham, England). Aquassure (New England Nuclear, Boston, Mass.) was used as liquid scintillation fluid.

Cell Isolation and Culture. EC from pig aortas taken at the slaughterhouse and washed in cold Krebs Ringer bicarbonate buffer (KRBB) containing 5 m*M* glucose were isolated after a 15-min treatment of the lumen of the vessels with a 0.05% collagenase (Worthington CLS type I, 202 U/ml) solution as already described (11).

Cells were cultured in RPMI 1640 medium containing 30 m*M* $NaHCO_3$ and 25 m*M* 2-/4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes) neutralized to pH 7.4. Culture medium was supplemented with 10% (v/v) fetal bovine serum (FBS; Seromed, Münich, W. Germany) throughout culture time.

Penicillin G (50 U) and streptomycin (50 μg) were added per milliliter medium. Freshly prepared amphotericin B (Fungizone, Squibb & Sons Inc., Princeton, N.J.) was added during the first 2 days of culture to a final concentration of 1 μg/ml medium. Cultures were kept at 37°C in a 5% CO_2–air atmosphere. When appropriate, Se-Met was added to the RPMI medium throughout the culture time at a final concentration of $2 \cdot 10^{-7}$ *M*. Cells were cultured in 35-mm-diameter petri dishes (Falcon, Oxnard, Calif.) coated with 0.1% gelatin (Difco Lab., Detroit, Mich.) (13).

EC were identified on morphological criteria (cobblestone appearance and monolayer growing pattern) and biochemical criteria by assessment of converting enzyme activity (14), which amounted to 3.6 ±0.4 nmole/mg prot/min, mean ± SEM of 27 experiments.

Experimental Procedure. *Enzyme reactions.* Experiments were performed on primary cultures, 1 to 4 days after confluence. The cells were washed twice with Krebs bicarbonate buffer supplemented with Hepes (KRBH) at 37°C and then 2 ml fresh KRBH was added. When the effect of O_2 intermediates, scavengers, and NaN_3 was tested, cells were preincubated with the appropriate agent for 5 min prior to the addition of XO, GO, or H_2O_2. The concentrations of the substrate for XO and GO were $2 \cdot 10^{-3}$ *M* hypoxanthine and 5 m*M* glucose, respectively.

The cells were generally maintained in the presence of the enzyme substrate system for 1 hr, except for the time–course experiments for which the duration of exposure ranged from 10 to 60 min. Experiments were carried out in a 5% CO_2–95% air atmosphere at 37°C. At the end of the incubation period, the medium was saved for the measurement of lactic acid dehydrogenase activity (LDH) and H_2O_2 and replaced by 2 ml fresh RPMI 1640 medium containing 10% (v/v) heat inactivated FBS. Cells were cultured for another 24-hr period, during which they received 1 μCi/ml [^{3}H]TdR. In experiments done to assess the duration of effects resulting from a 60-min exposure to O_2 intermediates, EC were maintained in culture for a 3-day period following the 60-min incubation in the presence of the enzyme–substrate system. For the measurement of the time–course of LDH release during the first hours after incubation, 200-μl samples were drawn from the culture medium at different time intervals and replaced by identical aliquots of fresh culture medium. At the end of the experiment, the culture medium was collected for LDH determination. Petri dishes were then washed three times with 2 ml cold KRBB and scraped in 1 ml potassium phosphate buffer (50 m*M*, pH 7.8) with a rubber policeman. Cells were sonicated twice for 10 sec at 4°C. Aliquots of the sonicate were kept at −20°C for subsequent DNA, protein, and LDH determinations.

An aliquot was also kept for the measurement of the radioactivity incorporated into DNA. It was precipitated with trichloroacetic acid as already reported (11) and the radioactive content of the TCA insoluble fraction was measured.

Initial rates of O_2^- production by the hypoxanthine + XO system, in the absence of cells, were measured according to Babior *et al.* (15) by following with a double-beam spectrophotometer at 550 nm the rate of

SOD inhibitable reduction of ferricytochrome *c* at 37°C in the presence of hypoxanthine ($2 \cdot 10^{-3}$ *M*) and different doses of XO in KRBH. O_2^- production was calculated using an extinction coefficient of $21.1 \cdot 10^3 M^{-1}$ cm^{-1} for the cytochrome *c* (red-ox) (16).

H_2O_2 production was assessed from the rate of decrease in scopoletin fluorescence induced by H_2O_2 in the presence of horseradish peroxidase (40 U/ml) according to the method of Root *et al.* (17). Excitation and emission wavelengths were 350 and 450 nm, respectively. The H_2O_2 content of the samples was determined using standard curve obtained under the same conditions with known amounts of H_2O_2.

Protein and DNA contents were measured through the methods of Lowry *et al.* (18) and Setaro and Morley (19), respectively.

LDH activity was evaluated according to the method described by Bergmeyer (20) and expressed in Wroblewski units (21). In sample containing FBS, a blank was always measured and its activity substracted.

Glutathione peroxidase was measured according to Paglia and Valentine (22) and catalase following the method of Lück (23).

Results. The initial rate of production of O_2^-, measured from the rate of reduction of cytochrome *c*, in the presence of $2 \cdot 10^{-3}$ *M* hypoxanthine in KRBH, pH 7.4 at 37°C, was 5.7, 10.9, 22.5, and 42.2 nmole O_2^-/ml/min for 10, 20, 40, and 80 mU XO/ml (n = 9–12). Addition of 200 U/ml catalase had no effect on the initial rate, but prevented the H_2O_2-related reoxidation of cytochrome *c* occurring later during the course of the reaction. From the rate of decrease of scopoletin fluorescence, the initial rate of production of H_2O_2 in the same reaction was 4, 10, and 18 nmole/ml/min for 10, 20, and 40 mU/ml XO, respectively.

No H_2O_2 could be detected in the presence of 200 U/ml catalase. Addition of 25, 50, and 100 mU/ml GO to KRBH supplemented with 5 m*M* glucose resulted in an initial rate of appearance of H_2O_2 of 5, 9, and 18 nmole H_2O_2/ml/min (n = 4–9). Thus, in terms of H_2O_2 production, 1 mU XO was roughly equivalent to 2.5 mU GO. The addition of 10% FBS to KRBH buffer resulted in a decrease in the rate of O_2^- production by 39 ± 12% (mean ± SE, n = 4). Hepes, on the other hand, had no apparent scavenging effect. All the subsequent experiments were done with KRBH buffer, pH 7.4.

Our first experiments with cultured EC were done to determine the time course of the cellular damage following a 60-min incubation in the presence of 40 mU/ml XO. Cytolysis, when assessed from LDH release into the culture medium (Fig. 1) (24), took place only gradually after the initial incubation period with the enzyme system. Since no further excessive LDH release followed this initial 24-hr observation period, we decided, for the subsequent experiments, to use a 24-hr period to study the overall cytotoxic effect of the enzyme reactions by determining not only LDH release, but also protein and DNA contents of the petri dishes. Because of our previous findings on the O_2-related inhibition of DNA synthesis (11), we also measured the incorporation of [^{3}H]TdR into DNA. Figures 2 to 5 present the dose–response curve obtained with different XO and GO activities in cells cultured with standard medium and with medium supplemented with Se-Met, the results being expressed as percentage of control value or in absolute terms for LDH release. In cells cultured with standard medium, TdR incorporation into DNA was the most sensitive index, being already significantly decreased in the presence of 10 mU XO ($P < 0.01$) or 50 mU GO ($P < 0.01$). The lowest dose of XO required to provoke significant changes in DNA content,

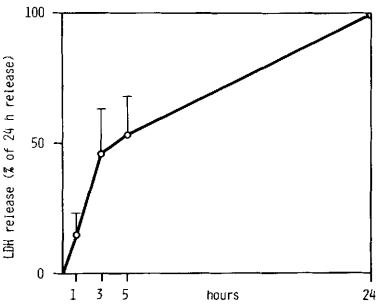

FIG. 1. Time course of LDH release after a 60-min exposure to 40 mU/ml xanthine oxidase. LDH release is expressed as percentage of the total LDH release over the 24-hr period (mean ± SE, n = 6).

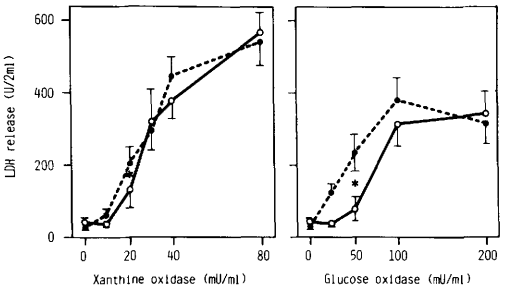

FIG. 2. Effects of various doses of xanthine oxidase and glucose oxidase on the amount of LDH released by endothelial cells cultured with standard medium (●) or with Se-Met supplemented medium (○). The incubation period in the presence of the enzyme system was 60 min. Asterisks represent statistically significant Se-Met-related differences (mean ± SE, n = 7–19). LDH content of cells cultured in standard medium and in Se-Met supplemented medium was 979 ± 53 and 901 ± 51 U/dish, respectively (mean ± SE).

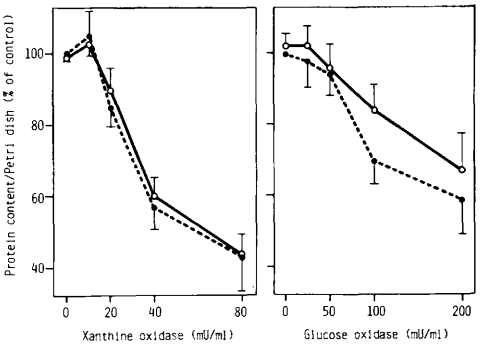

FIG. 4. Effects of various doses of xanthine oxidase and glucose oxidase on the protein content of petri dishes, 24 hr after a 60-min incubation in the presence of the enzyme system. No significant difference was found between endothelial cells cultured with standard medium (●) and those receiving Se-Met (○) (mean ± SE, n = 6–18).

protein content, and LDH release was 20 mU/ml, whereas the lowest doses of GO with a corresponding effect were 50 mU/ml (LDH release) and 100 mU/ml (DNA and protein content). The addition of Se-Met to culture medium resulted in a four-fold increase in glutathione peroxidase activity (from 30 ± 5 to 122 ± 19 U/mg protein, mean ± SEM, n = 7), without a concomitant increase in catalase activity (5.6 ± 0.6 vs 5.8 ± 0.7 U/mg protein, mean ± SEM, n = 7). Figures 2 to 5 also show that Se-Met treatment tended to be associated with a decreased sensitivity to the toxic effect of the reaction products generated by XO and GO. Although no significant difference related to Se-Met was found for the control conditions, and for the changes in protein content following exposure to the two enzyme systems, the changes affecting LDH release and [^{3}H]TdR incorporation into DNA were affected by Se-Met

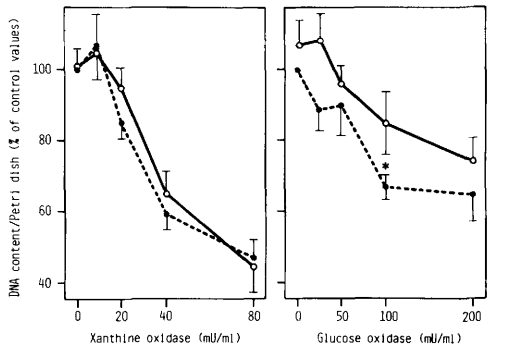

FIG. 3. Effects of various doses of xanthine oxidase and glucose oxidase on the DNA content of petri dishes, 24 hr after a 60-min incubation in the presence of the enzyme system. Asterisks represent statistically significant differences between endothelial cells cultured with standard medium (●) and those receiving Se-Met (○) (mean ± SE, n = 6–18).

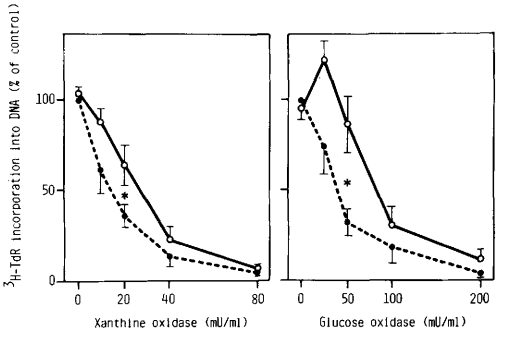

FIG. 5. Effects of various doses of xanthine oxidase and glucose oxidase on [^{3}H]TdR incorporation into DNA of endothelial cells cultured with standard medium (●) or with Se-Met supplemented medium (○). [^{3}H]TdR was added just after the incubation period in the presence of the enzyme system and its incorporation into DNA measured 24 hr later. Asterisks represent significant Se-Met-related differences (mean ± SE, n = 6–16).

treatment which offered a significant protective effect, with a shift to the right of the dose–response curve, especially in the range of low enzyme doses. For the DNA content, a significant Se-Met-related protective effect was found only in cells exposed to 100 mU/ml GO.

Various enzymes and scavengers were then used to determine the nature of the O_2 metabolite responsible for hypoxanthine–XO-related cytotoxicity. SOD, as well as benzoate and histidine, known as $OH^{\cdot}$ and $^1O_2$ scavengers, had no overall protective effect, whereas catalase blocked completely the cytotoxic effect of hypoxanthine–XO system (Table I). Catalase offered the same protection when EC were exposed to the glucose–GO system whereas boiled catalase was without any protective effect (data not shown). To test the validity of a possible explanation for the protective effect associated with increased glutathione-peroxidase activity, we decided to measure the rate of production of the H_2O_2 into the incubation medium in the presence of XO or GO. Figure 6 indicates that, in the presence of cells, there was a marked decrease in the cumulative amount of H_2O_2 formed over 60 min.

Furthermore, cells treated with Se-Met were capable of metabolizing H_2O_2 more efficiently than cells cultured in standard medium in presence of GO (50 mU): after 30 minutes, the amount of H_2O_2 present in the incubation medium of control cells and Se-Met-treated cells was 260 ± 41 and 170 ± 28 nmole respectively ($P < 0.01$) and, after 60 min, 279 ± 55 and 138 ± 37 nmole ($P < 0.01$) (mean ± SEM, $n = 6$). No evidence for the release of measurable amounts of catalase into the culture medium was found during an additional 60-min period following the initial incubation in the presence of the enzyme substrate systems. To have another assessment of the rate of degradation of the H_2O_2 present in the medium, we added a known amount of H_2O_2 (1 μmole) to the incubation medium. No difference could be found between Se-Met-treated and control cells. However, in the presence of $0.5 \cdot 10^{-3}$ *M* NaN_3, which inhibited catalase activity by 68%, but had no effect on glutathione peroxidase, there was again more H_2O_2 metabolized by Se-Met-treated cells: 406 vs 311 nmole for 30 min, 631 vs 524 nmole for 60 min ($P < 0.001$ for both series of experiments).

The next series of experiments was designed to determine the thresholds in terms of duration of exposure to the enzyme system and intensity of the reaction to elicit a cytotoxic effect.

Figure 7 shows that, with 40 mU/ml XO, a 60-min period was required to produce a significant change for all the indexes when

TABLE I. EFFECTS OF VARIOUS ANTIOXIDANT ENZYMES AND SCAVENGERS ON THE CYTOTOXICITY RESULTING FROM A 60-MIN INCUBATION IN THE PRESENCE OF 40 mU/ml XANTHINE OXIDASE

Experimental conditions	LDH release (U/2 ml)	DNA content	Protein content (% control values)	[^{3}H]TdR into DNA
Control	23 ± 9	100	100	100
+40 mU/ml XO	493 ± 112	60 ± 20	42 ± 16	10 ± 7
+40 mU/ml XO 200 U/ml catalase	65 ± 40	109 ± 30	96 ± 10	124 ± 38
+40 mU/ml XO 150 U/ml SOD	393 ± 120	68 ± 15	76 ± 9	16 ± 10
+40 mU/ml XO 1 m*M* histidine	607 ± 161	63 ± 19	52 ± 9	9 ± 13
+40 mU/ml XO 10 m*M* benzoate	415 ± 189	83 ± 42	66 ± 27	8 ± 12

Note. Values are means ± SD ($n = 4$–7).

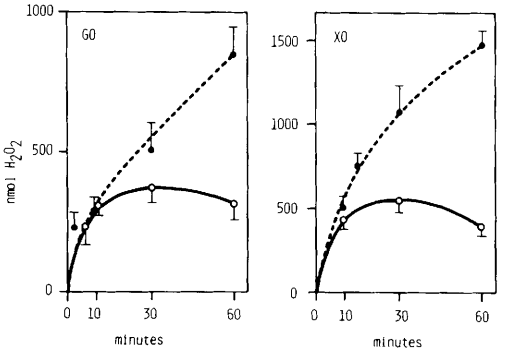

FIG. 6. H_2O_2 production under the action of 50 mU/ml glucose oxidase (left panel) or 40 mU/ml xanthine oxidase (right panel) in the absence (●) or presence (○) of endothelial cells (mean ± SE, n = 5–8).

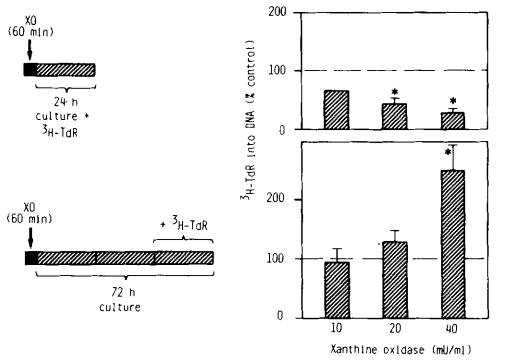

FIG. 8. Effects of various doses of xanthine oxidase on [^{3}H]TdR incorporation into DNA measured during the first 24 hr following the enzyme treatment or 2 days later. Results are expressed as percentage of values obtained under control conditions. Asterisks represent significant differences from control values (mean ± SE, n = 4–7).

tested during the subsequent 24 hr, except for [^{3}H]TdR into DNA which showed a significant reduction after a 30-min exposure to the enzyme system. A 60-min exposure to 50 mU/ml GO, on the other hand, had an effect similar to that of a 30-min incubation period in the presence of 40 mU/ml XO, namely, a decrease in DNA synthesis only.

Finally, experiments were done to test for a possible reversibility of the changes affecting DNA synthesis after the hypoxanthine–XO reaction. Figure 8 indicates that, although DNA synthesis was depressed in the first 24 hr after the enzyme reaction in a dose-dependent manner, there was, 2 days later, an increase in DNA synthesis. This increase was apparently related to the intensity of the previous cytotoxic effect, since most marked after an incubation in the presence of 40 mU/ml XO.

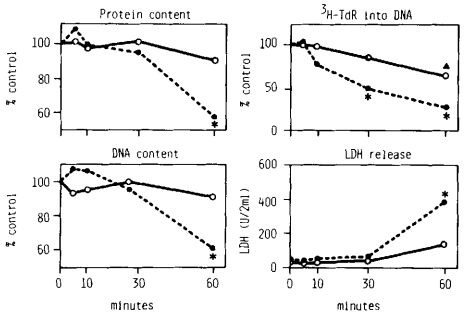

FIG. 7. Effects of the duration of exposure to 40 mU/ml xanthine oxidase (●) or 50 mU/ml glucose oxidase (○) on protein and DNA contents, [^{3}H]TdR incorporation into DNA, and LDH release. Asterisks represent significant differences from control values (n = 4–7).

Discussion. Many investigators have already used the substrate–XO system as well as various antioxidant enzymes and scavengers to test for involvement of the O_2 metabolites thus formed: O_2^-, $OH^\cdot$, H_2O_2, and $^1O_2$. This enzyme system provides a convenient way to assess the response of various cell populations to those O_2 metabolites which are also generated by activated inflammatory cells, PMN and monocytes. Thus, whether released by inflammatory cells or produced by enzyme reaction, O_2^- has been reported to cause red blood cell lysis (5, 25–28), whereas H_2O_2 has been shown to exert a cytotoxic effect on P388 lymphoma cells (29), red blood cells (30), T lymphocytes (31), and human cultured fibroblasts (32). The use of scavengers for $OH^\cdot$ suggested that $OH^\cdot$ radicals could be involved in certain conditions (33), but not in others (26, 29, 31).

The response of EC has received special attention, probably because of their strategic position and close contact with circulating inflammatory cells. Most investigators agree on the main role played by H_2O_2, whether generated by PMN or by the substrate–XO system, an appropriate amount of catalase

being able to give full protection (8–10). The present results confirm the predominant, but not necessarily exclusive, role of H_2O_2 in the development of EC injury, since all the effects could be blocked by catalase, but not by SOD or $OH^{\bullet}$ scavengers. Another supportive argument comes from the similarity of the effects resulting from the XO–hypoxanthine and GO–glucose reactions, which could be closely related to their capacity to generate H_2O_2. It should be mentioned, however, that for an equivalent amount of H_2O_2 produced XO tended to exert a more marked toxic effect. Se-Met addition also appeared to be slightly less effective against XO than against GO. Although the differences observed did not reach significance, this pattern might suggest that other O_2 intermediates might be involved in the development of injury. The use of various combinations of antioxidant enzymes and scavengers would be needed to confirm this assumption. The only apparent effect of SOD was the lesser reduction in protein content, which might depend on SOD addition, since this was already found in previous experiments done with control and O_2 exposed EC (34).

The toxic effect, whether resulting from XO or GO, was found to be crucially dependent on both the rate of production of H_2O_2, which is itself a function of the enzyme concentration, and on the duration of exposure. Nearly no toxic effect could be found on a 30-min exposure to 40 mU/ml XO, but marked cytolysis followed a 60-min exposure to the same dose of XO. On the other hand, a 60-min exposure to 50 mU/ml GO had approximately the same effect as a 30-min exposure to 40 mU/ml XO.

As already found for hyperoxia (11), the most sensitive index of cell toxicity was the incorporation of [^{3}H]TdR into DNA. Ten milliunits per milliliter of XO already significantly inhibited [^{3}H]TdR incorporation into DNA of cells cultured with standard medium. Otherwise, LDH release, an index of cytolysis closely correlated with ^{51}Cr release (35), showed a sharp sigmoid curve which suggests the presence of a narrow margin between a benign or reversible toxic effect and massive cytolysis. Once started after a lag period already noted by Simon *et al.* (32), the cytolytic process followed a gradual course over the next 24 hr. This time-course is important to consider when comparative analysis is made between the various experimental procedures used by other authors. A short follow up of the changes occurring after the initiation of the enzyme reaction might result in an important underestimation of its final effects.

Comparison between the effects observed by various investigators is also made more difficult because of other differences in the experimental conditions used: duration of exposure to XO and GO (from minutes to 6 hr); doses of XO and GO; presence of scavengers in the incubation medium; use of primary cultures or passages; culture on extracellular matrix or on plastic. All these elements can play a role in the amount of H_2O_2 formed and/or in the response of the cellular population tested.

The overall effect of Se-Met addition to culture medium and of the associated increase in glutathione peroxidase activity was to reduce the toxic effect of these enzyme reactions especially in the low dose range. The mechanism of this protective effect may be multiple. Detoxification of lipid peroxides formed by the reaction between polyunsaturated fatty acid and O_2 radicals is generally thought to be the main function of glutathione peroxidase (36). We tried to obtain such evidence by looking for the production of malondialdehyde by the thiobarbituric acid assay, but failed, perhaps because of inadequate sensitivity of the method used. An alternative role for glutathione peroxidase could be an increased rate of degradation of the H_2O_2 produced, as shown in the Results. H_2O_2 is known to diffuse freely across the cell membrane. Thus, glutathione peroxidase could act together with catalase to degrade more rapidly the H_2O_2 formed by enzyme systems as well as by activated PMN or monocytes. This role of glutathione peroxidase was already recognized as important in other cell populations to protect them against the toxic action of H_2O_2 (37–39). The detoxifying effect on lipid peroxides is not necessarily exclusive of the degradative action on H_2O_2, and both effects can account for the favorable effect resulting from the Se-induced increase in

glutathione peroxidase activity. When there is an excess of H_2O_2 formed, this protective mechanism is clearly saturated and loses its efficiency. The role of some of the glutathione red-ox cycle components (reduced glutathione, glutathione reductase) in the protection of EC against the toxic effects of H_2O_2 has recently been demonstrated by Harlan *et al.* (40). Our data confirm the importance of another enzymatic component of the antioxidant defense of EC, namely, glutathione peroxidase. An interesting difference can be noted with respect to the protective effect of increased G-Px activity against the H_2O_2-related inhibition of DNA synthesis when compared with its lack of effect against hyperoxia-induced decrease in [^{3}H]TdR incorporation into DNA (11). More has to be known on the nature of the hyperoxia- and H_2O_2-related inhibition of DNA synthesis before a satisfactory explanation can be offered. In fact, comparison with the intensity and the nature of the cellular damage associated with exposure to hyperoxia is also made difficult because of a lack of common denominator such as site and rate of O_2^- or H_2O_2 formation under these conditions when compared to that of H_2O_2 in the enzyme systems used. A finding common to both hyperoxic exposure and treatment with H_2O_2 generating systems has been, beside the sensitivity of DNA synthesis as an index of cytotoxicity, the occurrence of an apparent increase in DNA synthesis with the next 2–3 days following the injury (unpublished observation). This phenomenon which may be considered an expression of cell regeneration is of great interest since it is directly related to the problem of cell repair. The involvement of EC in the pathogenesis of various forms of pulmonary damage leading eventually to Adult Respiratory Distress Syndrome and of postischemic tissue injury (41) is now well recognized. Better knowledge on the factors capable of modulating the response of EC to injury or their repair process is of obvious clinical importance. Cultured EC provide a convenient tool to approach both problems.

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