CELL CALCIUM

Cell Calcium (1995) **17**, 307–315

Chemical hypoxia increases cytosolic Ca^{2+} and oxygen free radical formation

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Abstract — 'Chemical hypoxia' was produced in isolated rat hepatocytes. The cells were immobilized in agarose gel threads and perfused with Krebs-Henseleit bicarbonate buffer equilibrated with 95% O_2 + 5% CO_2 or 95% air + 5% CO_2. During 'chemical hypoxia', 2 mM KCN + 0.5 mM iodoacetate (CN-IAA) were added to the perfusate for 30 min. Cytosolic ionized Ca^{2+} (Ca_i^{2+}) was measured with aequorin, the formation of oxygen free radicals by lucigenin-enhanced chemiluminescence and cell injury by the rate of LDH released by the cells in the effluent perfusate. As soon as the cells were exposed to CN-IAA in the presence of 95% O_2 + 5% CO_2, Ca_i^{2+} increased rapidly to reach 1.5 μM within 10 min, and oxygen free radical formation increased 5-fold. The increase in LDH release was temporally delayed and occurred only during the recovery phase. The results were not significantly different when the cells were perfused with KHB equilibrated with 95% air + 5% CO_2, except that oxygen free radical formation increased 13-fold. These results suggest that both a rise in Ca_i^{2+} and a formation of reactive oxygen species could be responsible for the cell injury and the cell death induced by CN-IAA poisoning.

Many investigators have used 'chemical hypoxia' to study the problem of ischemic injury, although it is not a true model for anoxia [1–6]. The term 'chemical hypoxia' is used to describe experimental conditions in which the cells are exposed to cyanide and iodoacetate. 'Chemical hypoxia' is perhaps a misnomer since O_2 is always present which allows the formation of lethal amounts of oxygen free radicals in sharp contrast to true anoxia [3,4,7–9]. Cyanide and iodoacetate (CN-IAA) cause a rapid depletion of intracellular ATP comparable to that caused by anoxia, but the similarity between the two experimental conditions ends with the decline of ATP. Consequently, CN-IAA poisoning should not be considered a valid model for anoxia [4]. The difference between simple anoxia and CN-IAA poisoning is illustrated by the fact that the cell injury and the cell killing caused by CN-IAA poisoning is markedly reduced or delayed under anoxia, because there is obviously no formation of oxygen free radicals under anaerobic conditions [3,7–10]. Moreover, scavenging the free radicals or reducing their formation delays the occurrence of cell damage and the time of cell death induced by CN-IAA [3,7,10]. Mitochondrial dysfunction is primarily responsible for the formation of these oxygen free radicals, because mitochondrial inhibitors reduce the rate of hydroperoxide formation and of cell killing evoked by

CN-IAA [3,7]. In turn, free radicals may release Ca^{2+} from mitochondria through pyridine nucleotide oxidation and from endoplasmic reticulum through depletion of GSH and other protein thiols [11,12]. Furthermore, reactive oxygen species cause an activation of phospholipase A_2 which may lead to an influx of Ca^{2+} across the cell membrane [13]. In spite of the vast literature demonstrating a release of Ca^{2+} from intracellular Ca^{2+} pools induced by free radicals, there is presently no compelling data showing that CN-IAA poisoning increase cytosolic ionized Ca^{2+} (Ca_i^{2+}). It has been reported that 'chemical hypoxia' either does not increase Ca_i^{2+} [1,2,14–16] or causes a small delayed increase in Ca_i^{2+} [2,6,17] when assessed with fluorescent tetracarboxylic Ca^{2+} indicators. Only one article reports that in cultured murine hepatoma cells, 1 mM KCN and 10 mM IAA increase Ca_i^{2+} from 0.1 to 1.0 μM within 10 min [18]. In contrast, we have recently shown that simple anoxia causes a large increase in Ca_i^{2+} when measured with the Ca^{2+}-sensitive photoprotein aequorin [19,20]. The experiments described in the present article were undertaken to investigate whether aequorin is capable of detecting an increase in Ca_i^{2+} during CN-IAA poisoning. The results show that 2 mM KCN + 0.5 mM IAA increased Ca_i^{2+} from 0.1 to 1.5 μM within 10 min before any significant cell damage occurred, as assessed by LDH release from the cells. In addition, CN-IAA poisoning caused a large formation of oxygen free radicals as measured by lucigenin-enhanced chemiluminescence.

Materials and methods

Hepatocytes

Freshly isolated hepatocytes were prepared from adult male Sprague-Dawley rats weighing between 150–250 g. The cells were isolated from fed animals with collagenase (Sigma type IV, Sigma Chemicals, St Louis, MO, USA) and incubated in standard Krebs-Henseleit bicarbonate buffer (KHB) equilibrated with 95% O_2 + 5% CO_2 as described previously [19,20] or with 95% air + 5% CO_2. Cell viability was assessed by trypan blue exclusion. Immediately after isolation, the cell viability ranged between 85–95%, and there were some cell debris and a few occasional non-parenchymal cells. The cells were then layered on a solution consisting of 33% Percoll in 150 mM NaCl and 10 mM HEPES at pH 7.4 and were centrifuged for 10 min at 12,000 *g*. At this stage, cell viability averaged 93% without any cell debris or nonparenchymal cells. The equivalent of 0.25 ml of packed cells was resuspended in 0.25 ml KHB; 0 5 ml of 1.4 % agarose in KHB was added to the 0.5 ml cell suspension and the 1.0 ml cell-agarose suspension was aspirated in a 1 ml tuberculin syringe, forced through a 0.5 mm catheter coiled in a 4°C water bath containing crushed ice to obtain cells imbedded in agarose gel threads of 0.5 mm diameter. The threads were placed in a 10 x 40 mm cuvette containing 2 ml KHB and the cells were perfused at 37°C at a rate of 0.6 ml/min with KHB containing 1.3 mM Ca^{2+} and 5 mM glucose. Each perfusion chamber contained 35 ± 1 mg of cell protein.

Cytosolic free calcium

Ca_i^{2+} was determined using aequorin loaded cells, the photoprotein being incorporated by gravity loading [21], i.e. 50 g for 60 s (500 rpm in a standard IEC centrifuge). The aequorin loaded cells cast in agarose gel threads were placed in the cuvette of an aequorin luminescence photometer and perfused at a rate of 0.6 ml/min with KHB at 37°C, as previously described [19,20]. The light signal was calibrated assuming an intracellular free Mg^{2+} concentration of 0.5 mM in control conditions and a Mg_i^{2+} of 1 mM during chemical hypoxia since such an increase has been reported under these conditions [5]. The aequorin luminescence signal was integrated throughout the experiment, and the calculation of Ca_i^{2+} at each time point was referred to the final maximal luminescence (L_{max}) + the integrated amount of the photoprotein consumed after that point [19,20]. Recording of the aequorin luminescence was made on a MacLab–/4 data interface module (AD Instruments Inc., Milford, MA, USA).

Measurement of oxygen free radicals

Free radical formation was measured by the enhanced chemiluminescence emitted by the perfused

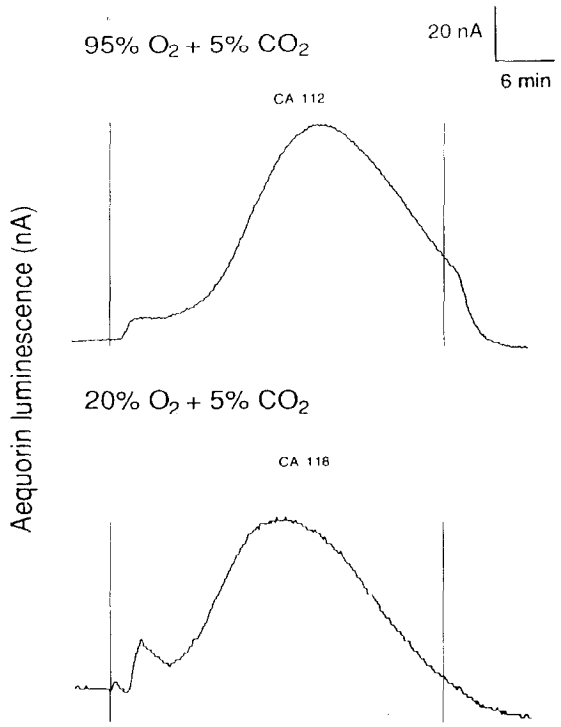

Fig. 1 Original tracings of the aequorin luminescence recorded to measure Ca_i^{2+} in freshly isolated rat hepatocytes perfused with KHB equilibrated with 95% O_2 and 5% CO_2 (upper tracing) or with 95% air and 5% CO_2 (lower tracing) and exposed for 30 min to 2 mM KCN and 0.5 mM iodoacetate. The experimental period of 'chemical hypoxia' is indicated by the vertical lines. The 1 min delay between the lines and the changes in aequorin luminescence is due to the dead time for the perfusate to reach the cells. These recordings are representative of 6 experiments with 95% O_2 and 5% CO_2 and 5 experiments with 95% air and 5% CO_2.

hepatocytes cast in gel threads. Superoxides ($O_2^{\bullet}$)were detected by lucigenin-enhanced chemiluminescence by adding 10^{-6} M lucigenin to the perfusate, as previously described [9]. The chemiluminescence was measured in the same photometers used for aequorin luminescence. The high voltage supply to the photomultiplier was set at its optimal signal-to-noise ratio, i.e 1200 V. Under these conditions, the background or dark current of the photometers was in the range of 0.1–0.3 nA. Recording of the enhanced chemiluminescence was made on a MacLab–/4 data interface module (ADInstruments Inc.).

Lactic dehydrogenase (LDH) release

Cell injury was monitored by measuring the rate of LDH release in the effluent perfusate before, during and after CN-IAA poisoning.

Protocol for CN-IAA poisoning

Hepatocytes were first perfused for 30 min in control KHB saturated with 95% O_2 and 5% CO_2 or with 95% air and 5% CO_2. During the 30 min experimental period, 2 mM KCN + 0.5 mM IAA were added to the perfusate. After the 30 min experimental period, the cells were perfused again with control KHB for 30 min.

Statistics

Statistically significant differences between groups were determined using either Student's t test or ANOVA when appropriate. A P value less than 0.05 was considered to be significant.

Results

Experiments performed under 95% O_2 and 5% CO_2

Control conditions. During the control period, Ca_i^{2+} was 136 ± 14 nM, and the rate of LDH release was 93 ± 7 mU/min. The lucigenin-enhanced chemiluminescence which detects $O_2^{\bullet}$ generation was 5.7 ± 0.9 nA which was 30 times background. Under such conditions, the rate of $O_2^{\bullet}$ formation remained low and constant for several hours. Bolaan and Ulvik showed that, in vitro, 100 µM xanthine and 20 mU xanthine oxidase generate 11 nmol superoxide/min [22]. In the same in vitro conditions, we measured a lucigenin-enhanced chemiluminescence of 10.4 ± 0.9 nA [9]. From these values, we have calculated that, under 95% O_2 and 5% CO_2, the steady state rate of superoxide ($O_2^{\bullet}$) formation was 170 pmol $O_2^{\bullet}.min^{-1}.mg^{-1}$ cell protein. The relevance of this value obtained in isolated hepatocytes under 95% O_2 to the superoxide formation by the intact liver in vivo with

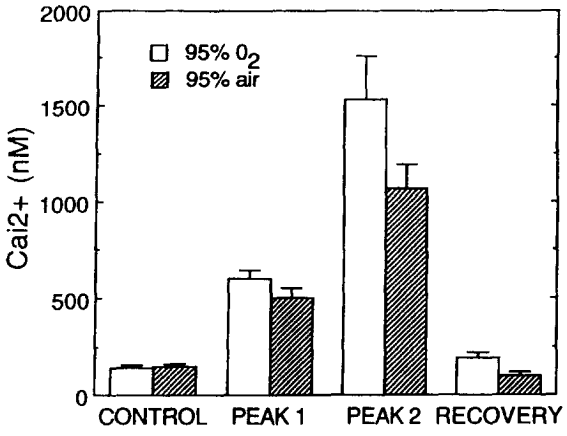

Fig. 2 Effect of 2 mM KCN and 0.5 mM iodoacetate on Ca_i^{2+} calculated from the aequorin luminescence tracings. The bars represent the mean ± SE of 6 experiments with 95% O_2 and 5% CO_2 and 5 experiments with 95% air and 5% CO_2.

a pO_2 of 40 torr is difficult to assess, but these calculations provide an order of magnitude for the rate of $O_2^{\bullet}$ production in isolated cells in vitro, a value that can be compared to the rate obtained in similar experiments performed by other investigators.

Effect of CN-IAA. Figure 1 shows that, when 2 mM KCN and 0.5 mM iodoacetate were added to the perfusate, the aequorin luminescence increased in two stages: it rose briefly within 2–5 min as a first transient peak then increased to a new higher level in 10–15 min. Thereafter, the aequorin luminescence declined slowly. This is similar to what we observed during true anoxia [19,20]. When, after 30 min, the perfusate with CN and IAA was replaced by a perfusate without inhibitors, the aequorin luminescence returned rapidly to the control level and remained low throughout the recovery period. Figure 2 shows that in 5 separate experiments, the first Ca_i^{2+} peak rose to 598 ± 45 nM ($P < 0.01$), and the second to 1528 ± 230 nM ($P < 0.01$).

Figure 3 presents representative tracings of the lucigenin-enhanced chemiluminescence obtained when the cells were exposed to CN-IAA. Figure 4 shows that the lucigenin-enhanced chemiluminescence increased progressively throughout the experimental period to reach a maximum of 32 ± 8 nA ($P < 0.01$) which represents a rate of $O_2^{\bullet}$ formation approximately 5.6 times higher than control, or 0.9 nmol $O_2^{\bullet}.min^{-1}.mg^{-1}$ cell protein. During the recovery period, $O_2^{\bullet}$ production assessed by lucigenin chemiluminescence kept increasing for at least 30 min.

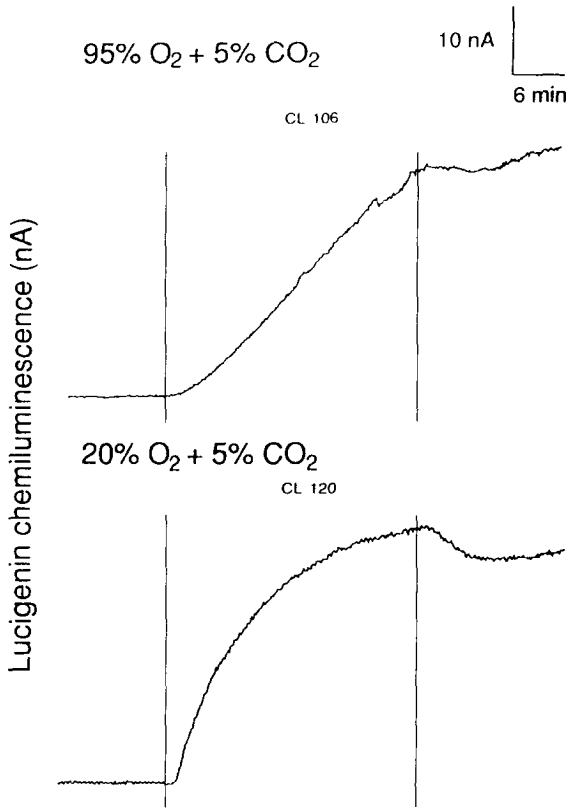

Fig. 3 Original tracings of the lucigenin-enhanced chemiluminescence recorded to measure superoxide formation in freshly isolated rat hepatocytes perfused with KHB equilibrated with 95% O_2 and 5% CO_2 (upper tracing) or with 95% air and 5% CO_2 (lower tracing) and exposed for 30 min to 2 mM KCN and 0.5 mM iodoacetate. The experimental period of 'chemical hypoxia' is indicated by the vertical lines. The 1 min delay between the lines and the changes in lucigenin-enhanced chemiluminescence is due to the dead time for the perfusate to reach the cells. These recordings are representative of 5 experiments with 95% O_2 and 5% CO_2 and 4 experiments with 95% air and 5% CO_2.

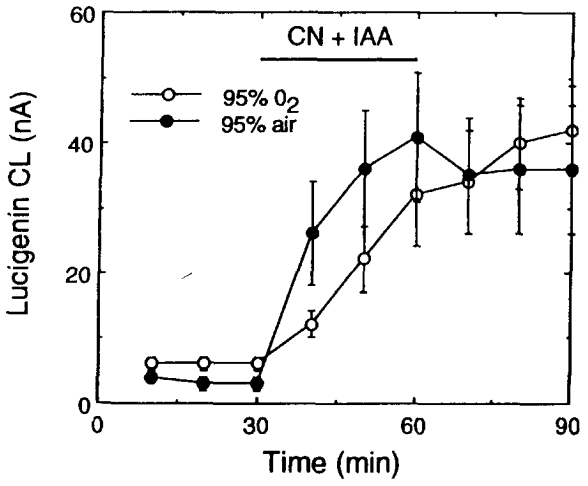

Fig. 4 Effect of 2 mM KCN and 0.5 mM iodoacetate on the lucigenin-enhanced chemiluminescence from freshly isolated rat hepatocytes perfused with KHB equilibrated with 95% O_2 and 5% CO_2 or with 95% air and 5% CO_2. Each point represents the mean ± SE of 5 experiments with 95% O_2 and 5% CO_2 and 4 experiments with 95% air and 5% CO_2.

Finally, Figure 5 shows that the rate of LDH released by the cells barely increased during the 30 min of CN-IAA exposure from a control of 93 ± 7 to 144 ± 34 mU/min ($P < 0.05$). During the recovery period, however, LDH release increased and reached 353 ± 74 mU/min at 90 min. It is clear from these results, that the increase in Ca_i^{2+} and the rise in chemiluminescence occurred at a time when LDH release was barely above control values. Moreover, the increased $O_2^{\bullet}$ production assessed by lucigenin chemiluminescence preceded the cell injury measured by the rise in LDH release. These two sets of results demonstrate that the rise in Ca_i^{2+} and the increase in free radical formation were not a consequence of cell injury or of cells dying from CN-IAA poisoning.

Experiments performed under 95% air and 5% CO_2

Hyperoxic conditions may possibly result in the cellular hyperproduction of potentially toxic reactive oxygen species [23,24]. To eliminate the possibility that our results may have been influenced by the high pO_2 present in media equilibrated with 95% O_2 and 5% CO_2, we repeated these experiments with perfusate equilibrated throughout with 95% air and 5% CO_2. Under control conditions, the resting Ca_i^{2+} was not different in the two groups: 136 ± 14 nM in 95% O_2 compared to 142 ± 21 nM in 95% air. Superoxide formation assessed by lucigenin-enhanced chemiluminescence was higher in 95% O_2 than in 95% air: 5.7 ± 0.9 compared to 3.0 ± 0.8 nA respectively, but the difference is not statistically significant. On the other hand, the rate of LDH release, under control conditions in 95% air was twice as high as in 95% O_2: 176 ± 46 vs 93 ± 7 mU/min respectively ($P < 0.05$).

When the cells were exposed to CN-IAA in 95% air and 5% CO_2, there was no significant difference in the increase in Ca_i^{2+}, in $O_2^{\bullet}$ production and in LDH release when compared to cells exposed to 95% O_2 and 5% CO_2. The second Ca_i^{2+} peak was slightly less in 95% air: 1062 ± 130 vs 1528 ± 230 nM (Fig. 2), whereas $O_2^{\bullet}$ production and LDH release were slightly greater (Figs 4 & 5), but the dif-

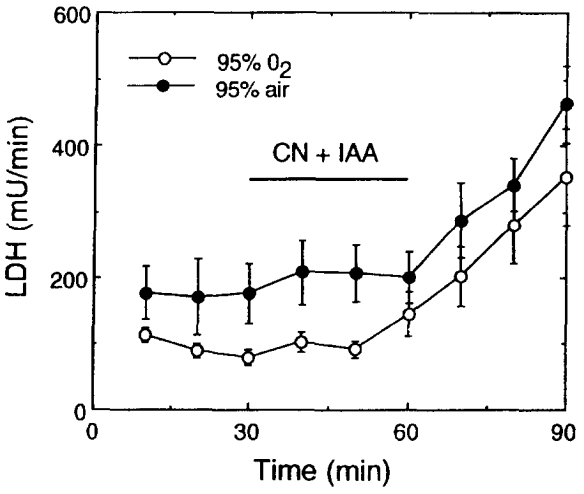

Fig. 5 Effect of 2 mM KCN and 0.5 mM iodoacetate on the rate of LDH release from freshly isolated rat hepatocytes perfused with KHB equilibrated with 95% O_2 and 5% CO_2 or with 95% air and 5% CO_2. Each point represents the mean ± SE of 10 experiments with 95% O_2 and 5% CO_2 and 6 experiments with 95% air and 5% CO_2.

ferences measured during and after 'chemical hypoxia' were not statistically significant.

Discussion

From these experiments, it is clear that CN-IAA poisoning caused an early and large increase in Ca_i^{2+} when measured with aequorin in rat hepatocytes. This is in marked contrast with previous reports which show either no increase in Ca_i^{2+} when measured with fluorescent dyes, or a late and relatively small increase after 40–80 min of exposure to CN-IAA, when the cell viability has declined 50% or more, and just before the Ca^{2+} indicator is leaking out of the cells [1,2,6,14–17]. It is not clear why fluorescent Ca^{2+} indicators do not detect a rise in Ca_i^{2+} under the conditions of anoxia or 'chemical hypoxia'. It is possible that Fura–2 is not restricted to the cytosol. Many investigators have shown that, in some cells, Fura–2 is taken up by mitochondria [25–30] and by other subcellular organelles [31]. As a result, DeFeo et al. proposed that the mixed signals from a rising Ca^{2+} activity in the cytosol and from a declining Ca^{2+} concentration in the intracellular organelles may blur or mask a real increase in Ca_i^{2+} [31]. However, Nieminen et al. reported that, in cultured rat hepatocytes, only 11 % of the incorporated Fura–2 was localized to lysosomes and other endomembrane compartments and 6% to mitochondria and that the Ca^{2+} indicator was predominantly located in the cytosol [32]. EGTA and its derivatives, Quin–2 and Fura–2 have been shown to inhibit Na^+/Ca^{2+} exchange operating in the reverse mode [33–37], which may inhibit Ca^{2+} influx through the plasma membrane believed to cause the large rise in Ca_i^{2+} evoked by anoxia [19,20]. Ca^{2+}-EGTA, Ca^{2+}-Quin–2 and Ca^{2+}-Fura–2 complexes have been shown to act as a substrate for the Ca^{2+}-ATPase dependent Ca^{2+} transporter and to activate the Ca^{2+} pump [38]. Finally, when aequorin is incorporated together with Quin–2 in a cell population, or with Fura–2 in a single cell, the peak Ca_i^{2+} detected with the fluorescent dyes is respectively 10 and 100 times smaller than that measured with aequorin [39,40]. Similar discrepancies between the results obtained with aequorin and those measured with Fura–2 exist in the literature regarding the effects of anoxia on Ca_i^{2+} in liver and kidney cells. Whereas our laboratory found that anoxia causes large increases in Ca_i^{2+} when measured with aequorin [19–21,41,42], other groups using Fura–2 were not able to detect any increase in Ca_i^{2+} [1,2,14–16,43].

We have recently shown that $O_2^{\bullet}$ formation by perfused hepatocytes can be measured in real time by lucigenin-enhanced chemiluminescence [9]. The present experiments show that CN-IAA increases the formation of reactive oxygen species 5–7 fold as previously reported [3,7]. Since KCN inhibits catalase, it is possible that the increased chemiluminescence may be caused by a decreased detoxication of endogenously generated oxidant. Indeed, the concentration of $O_2^{\bullet}$ depends on its rate of formation on the one hand and on its rate of dismutation to H_2O_2 on the other, and it is difficult to distinguish between an increased $O_2^{\bullet}$ formation and a decreased elimination. In control conditions, however, the generation of $O_2^{\bullet}$ and H_2O_2 is very small and, even if H_2O_2 was not detoxified by catalase, it would be detoxified by various peroxidases or transformed to $HO^{\bullet}$ by the Fenton reaction. We have already shown that blocking the Fenton reaction with low concentrations of deferroxamine which chelate iron during $O_2^{\bullet}$ generation induced by post anoxic reoxygenation, prevents $HO^{\bullet}$ generation from H_2O_2 and eliminates lipid peroxidation assessed by malondialdehyde formation without increasing $O_2^{\bullet}$ assessed by lucigenin-enhanced chemiluminescence [10]. Therefore, we think it is unlikely that the increase in $O_2^{\bullet}$ induced by chemical hypoxia is caused by an inhibition of catalase. Their formation did not occur before the rise in Ca_i^{2+} indicating that the increase in Ca_i^{2+} is not necessarily the consequence of oxygen free radical formation. Finally, the increase in the rate of LDH release was temporally delayed compared to the rise in Ca_i^{2+} and to the formation of free radical. This eliminates the possibility that the rise in Ca_i^{2+} and the formation of radicals were a consequence of cell injury or of cell death. During the recovery period, Ca_i^{2+} returned to its control

level in a few minutes. In contrast, the formation of reactive oxygen species and the release of LDH kept increasing during the 30 min of recovery. The formation of these free radicals was probably one of the causes of the cell injury. Indeed, the cell killing caused by CN-IAA poisoning is known to be markedly reduced and delayed under anoxia which prevents the formation of oxygen free radicals [3,4,6,7]. Furthermore, scavenging the free radicals or reducing their formation delays the occurrence of cell damage and the time of cell death induced by CN-IAA: mannitol, a free radical scavenger, deferroxamine, an inhibitor of the Haber-Weiss reaction that results in the ultimate formation of hydroxyl radicals, and allopurinol, an inhibitor of xanthine oxidase which is another source of oxygen free radicals, all decrease cell injury and delay cell death caused by CN-IAA [3,7,10].

It has been proposed that oxygen free radicals trigger phospholipid hydrolysis and activate phospholipase A_2 which could lead to an influx of Ca^{2+} across the cell membrane [13,17]. These free radicals could also release Ca^{2+} from mitochondria through pyridine nucleotide oxidation and from endoplasmic reticulum through depletion of GSH and other protein thiols [11,12]. However, the first peak in Ca_i^{2+} occurred earlier than any significant rise in $\dot{O}_2^-$ formation and therefore it may not be a consequence of their release. The increased in Ca_i^{2+} is probably caused, at least in part, by a severe disruption of mitochondrial function by CN-IAA [4]. Under aerobic conditions, KCN collapses the mitochondrial ΔpH, decreases $\Delta\psi$, causes an excessive phosphate loading, a loss of osmotic regulation of these organelles and a release of Ca^{2+} from mitochondria [4]. In addition to the failure of the mitochondrial protonmotive force, the resulting rise in Ca_i^{2+} can cause cell injury and cell death [19,20]. it can alter hepatocyte cytoskeletal structure because Ca^{2+} and its associated binding proteins play a pivotal role in regulating cytoskeletal structures and causes the formation of blebs. But the most likely cause responsible for cell injury is the activation of hydrolytic enzymes: Ca^{2+}-dependent proteases, endonucleases and phospholipases are all activated by a rise in Ca_i^{2+}. Ca^{2+}- and thiol-sensitive proteases initiate intracellular protein degradation. Endogenous endonucleases cause DNA degradation and nuclear fragmentation. Phospholipase A_2 attacks the integrity of plasma membrane and organelle membranes which may aggravate the disruption of intracellular Ca_i^{2+} homeostasis.

Under hyperoxic conditions, the high oxygen tension leads to an overproduction of superoxides, of hydroperoxides and of other potentially toxic reactive oxygen species, although the partial pressure of oxygen in media exposed to 95% O_2 (502 mm Hg) is only 3 times greater than that of media incubated with 95% air (166 mm Hg) [23,24]. However, the viability of freshly isolated hepatocytes is not affected by this increased production of reactive oxygen species for at least 6 h [24]. Cell injury, assessed by loss of intracellular K^+ and LDH leakage, occurs only after 6 h of incubation; during the first 6 h, there is no difference in cell viability between 95% O_2 and 95% air. After 6 h, a 95% O_2 atmosphere is indeed toxic to hepatocytes in suspension [24]. In our experiments and under control conditions, the rate of superoxide production assessed by lucigenin-enhanced chemiluminescence was twice as high under 95% O_2 as under 95% air: 6.0 ± 1.1 vs 3.0 ± 0.9 nA, but the difference was not statistically significant. On the other hand, the rate of LDH release was twice as high in 95% air as in 95% O_2: 176 ± 46 vs 79 ± 12 mU/min ($P < 0.05$). Evidently, there is no correlation between the low rate of $\dot{O}_2^-$ production and the low rate of LDH release under control conditions. Moreover, there was no difference in the resting level of Ca_i^{2+} of both groups: 136 ± 14 nM under 95% O_2 and 142 ± 21 nM under 95% air. When the cells were exposed to CN-IAA, there was no significant difference either in the rise in Ca_i^{2+}, the rise in lucigenin-enhanced chemiluminescence and in the increase in the rate of LDH release, whether the cells were exposed to 95% O_2 or 95% air. We conclude that our results showing that 'chemical hypoxia' increased Ca_i^{2+} and free radical formation was not caused by hyperoxic conditions.

In conclusion, CN-IAA poisoning increased Ca_i^{2+} and caused the formation of oxygen free radicals. Cell injury assessed by LDH release occurred after the rise in Ca_i^{2+} and the formation of radicals. Apparently, both free radicals and Ca^{2+} are involved

in the sequence of events leading to cell injury. The question of which is most important is unclear but both, given time, are quite capable of killing the cells.

Acknowledgement

This work was supported by NIH grant R01 DK 46935-01.

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Received :
Revised :
Accepted :