Increased flux of free radicals in cells subjected to hyperthermia: detection by electron paramagnetic resonance spin trapping

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Abstract It has been hypothesized that hyperthermia promotes oxygen-centered free radical formation in cells; however, to date there is no direct evidence of this heat-induced increase in oxygen free radical flux. Using electron paramagnetic resonance (EPR) spin trapping, we sought direct evidence for free radical generation during hyperthermia in intact, functioning cells. Rat intestinal epithelial cell monolayers were exposed to 45°C for 20 min, after which the nitrone spin trap 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) was added. Compared to control cells at 37°C, heat-exposed cells had increased free radical EPR signals, consistent with the formation of DMPO/$^{\cdot}$OH ($a^N = a^H = 14.9$ G). These findings indicate that heat increases the flux of cellular free radicals and support the hypothesis that increased generation of oxygen-centered free radicals and the resultant oxidative stress may mediate in part, heat-induced cellular damage.

Key words: Hyperthermia; Free radical; Electron paramagnetic resonance; Spin trapping; Heat injury; Oxidative stress

1. Introduction

Reactive oxygen species (ROS) have been demonstrated to be mediators of cellular injury. Exposure to an oxidant stress induces a pattern of gene expression somewhat similar to that observed following hyperthermia, suggesting a mechanistic link between the two stresses [1,2]. A number of biochemical and physiological events associated with hyperthermia can potentially promote free radical formation. Mitochondria, one of the main sources of ROS in cells [3], undergo a temperature-dependent uncoupling during increases in temperature [2,4]. Using isolated skeletal muscle mitochondria subjected to heat stress, this uncoupling has been associated with increased superoxide formation, using a cytochrome *c* assay [2]. Hyperthermia also increases the conversion of the enzyme xanthine dehydrogenase to the oxidase form, an important source of oxygen-derived free radicals [5].

Additional evidence supporting heat-induced generation of ROS has been provided using other indirect indices of oxidative stress. Glutathione, an electron donor for the antioxidant enzyme glutathione peroxidase (GPx), increases rapidly in cells exposed to elevated temperatures, while depletion of intracellular GSH significantly sensitizes cells to a thermal stress [6,7]. In addition, hyperthermic liver perfusion increases the efflux of glutathione disulfide (GSSG) [8]. Both of these observations on GSH and GSSG are indicative of oxidative stress. If antioxidant enzymes are in part regulated by their individual substrates, then an increase in ROS generation should be reflected in the activity of antioxidant enzymes. Indeed, several investigators have shown marked increases in antioxidant enzyme activity following heat exposure [9,10]. Similarly, modulation of antioxidant enzymes should affect thermal sensitivity. Lowering SOD enzyme levels results in a significant reduction in thermal resistance [11], while overexpression of MnSOD by stable transfection provides cellular resistance against the cytotoxic effects of heat [12]. Based on these findings it has been postulated that the cellular damage and/or toxicity associated with hyperthermia is mediated in part by an increased flux of ROS.

Although there are indirect data suggesting that ROS are generated during hyperthermia, to date however, there is no direct evidence for oxygen-centered radical formation from cells. Based on evidence suggesting that alterations in intestinal epithelial permeability are important in the pathogenesis of heat injury [13,14], the non-transformed rat intestinal epithelial cell (IEC) was chosen as a model in which to examine free radical generation associated with heat stress. We hypothesized that hyperthermia would produce an increased flux of EPR detectable free radicals.

2. Materials and methods

2.1. Cell culture

The non-transformed rat intestinal epithelial cell line (IEC; American Tissue Culture Collection) was grown as monolayers in Dulbecco's minimal essential medium (DMEM) plus 10% fetal bovine serum in a 37°C, 5% CO_2 incubator. Cells were maintained as an exponentially growing culture by passage every 2–4 days.

2.2. Heat and oxidant exposure

After growing to confluency, IEC monolayers were washed twice with PBS and then placed in a 45°C waterbath for 20 min. Menadione (100 μM) was used as a standard to generate superoxide inside cells. PBS was used for all treatments and controls. The nitrone spin trap 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) was used for detection of free radicals. Following heat or menadione exposure, DMPO was added to a final concentration of 50 mM. Samples of the supernatant (500 μl) were collected 60 s after the addition of DMPO and frozen in liquid N_2 until analysis.

2.3. EPR measurements

Electron paramagnetic resonance spectra were collected using a Bruker ESP-300 EPR spectrometer equipped with a TM_{110} cavity and appropriate aqueous flat cell. Instrument settings were: sweep rate 80 G/84 s; modulation amplitude 1.0 G; nominal microwave power 40 mW; time constant 82 ms. Hyperfine splitting constants were measured directly from spectra.

3. Results and discussion

To examine whether hyperthermia generates an increased flux of free radicals in intact, viable cells, EPR spin trapping

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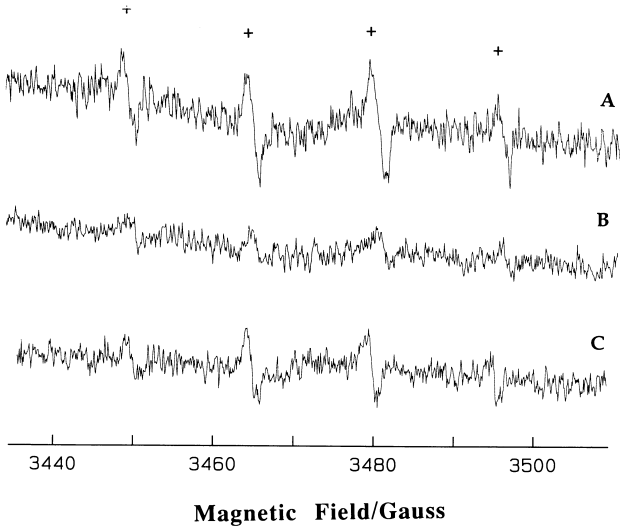

Fig. 1. Intestinal epithelial cell (IEC) monolayers subjected to hyperthermia produce free radicals as seen by EPR observation of DMPO/•OH. IEC were exposed to: (A) 45°C waterbath for 20 min; (B) 37°C for 20 min; (C) Menadione (100 μM) for 20 min. Spectra presented are the composites of five (A and B) independent experiments. Spectrum C is representative of one independent experiment. All spectra have been normalized for total number of scans. + indicates the spectral lines of 1:2:2:1 quartet ($a^N = a^H = 14.9$ G) of the DMPO/•OH adduct.

studies were performed using the spin trap DMPO to detect oxygen-centered radicals. Cells exposed to hyperthermia for 20 min demonstrated free radical signals in the presence of DMPO, Fig. 1A. In contrast to heated cells, cells maintained at 37°C exhibited little EPR signal, Fig. 1B. EPR spectra consisted of a 1:2:2:1 quartet pattern with hyperfine splitting constants ($a^N = a^H = 14.9$ G) consistent with the DMPO/•OH adduct [15]. In the absence of cells no differences in the DMPO/•OH adduct signal intensity were observed between heated and control conditions (not shown). These data demonstrate that hyperthermia increases the flux of free radicals in cells.

The DMPO/•OH signal observed in cells subjected to hyperthermia can arise from either the reaction of $O_2^{\bullet-}$ with DMPO to form DMPO/•OOH, which can be converted to DMPO/•OH, or the direct trapping of HO• [16]. DMPO can diffuse across the cell membranes to react intracellularly with the highly reactive •OH radical ($k = 3.4 \times 10^9$ M^{-1} s^{-1} [17]). However, in cells the DMPO/•OH adduct formed is rapidly metabolized, making EPR detection difficult [18]. Alternatively, $O_2^{\bullet-}$ generated within the cell may diffuse across the cell membrane to generate extracellular DMPO adducts [18]. Although DMPO reacts much slower with $O_2^{\bullet-}$ (k_{obs}(pH 7.4) = ~30 M^{-1} s^{-1} [17]) than HO•, the relatively long half-life and low reactivity of $O_2^{\bullet-}$ implies effective interaction with DMPO [18], suggesting that the DMPO/•OH adduct observed in the present study may correspond to trapping of $O_2^{\bullet-}$ generated during hyperthermia. To test this possibility, we used menadione as a tool. Menadione is well known to redox cycle and thus produce $O_2^{\bullet-}$ in cells [19]. When cells were exposed to menadione in the presence of DMPO at 37°C, DMPO/•OH was observed (Fig. 1C), parallel to the observation with hyperthermia, Fig. 1A.

The precise location and mechanism(s) of increased formation of oxygen-derived free radicals during heat stress remains unclear. However, consistent with the conclusion that the DMPO/•OH adduct observed corresponds to an increased generation of $O_2^{\bullet-}$ are findings that the mitochondrial electron transport chain may be an important source of $O_2^{\bullet-}$ during hyperthermia [2,4]. Generation of $O_2^{\bullet-}$ has also been closely associated with the univalent reduction of oxygen by mitochondrial semiquinones [3]; semiquinone radicals have been directly observed in the portal blood of hyperthermic rats by EPR spectroscopy [20]. Moreover, the elevation of the mitochondrial superoxide dismutase (MnSOD) in cells results in a remarkable increase in thermotolerance [12]. These observations coupled with the data presented here support the hypothesis that increased generation of free radicals and associated oxidants may mediate, in part, heat-induced cellular damage.

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References

[1] Schiaffonati, L., Rappocciolo, E., Tacchini, L., Cairo, G. and Bernelli-Zazzera, A. (1990) J. Cell. Physiol. 143, 79–87.
[2] Salo, D., Donovan, C. and Davies, K. (1991) Free Radic. Biol. Med. 11, 239–246.
[3] Chance, B., Sies, H. and Boveris, A. (1979) Physiol. Rev. 59, 527–604.
[4] Brooks, G.A., Hittelman, K.J., Faulkner, J.A. and Beyer, R.E. (1971) Am. J. Physiol. 220, 1053–1059.
[5] Skibba, J.L., Stadnicka, A., Kablfleisch, J.H. and Powers, R.H. (1989) J. Biochem. Toxicol. 4, 119–125.
[6] Mitchell, J. and Russo, A. (1983) Radiat. Res. 95, 471–485.
[7] Freeman, M.L., Spitz, D.R. and Meredith, M.J. (1990) Radiat. Res. 124, 288–293.
[8] Abdalla, E., Cata, M., Guice, K., Hinshaw, D. and Oldham, K. (1990) J. Surg. Res. 48, 291–296.
[9] Loven, D.P., Leeper, D.B. and Oberley, L.W. (1985) Cancer Res. 45, 3029–3033.
[10] Hass, M. and Massaro, D. (1988) J. Biol. Chem. 263, 776–781.
[11] Omar, R.A., Yano, S. and Kikkawa, Y. (1987) Cancer Res. 47, 3473–3476.
[12] Li, J.J. and Oberley, L.W. (1997) Cancer Res. 57, 1991–1998.
[13] Gathiram, P., Wells, M.T., Raidoo, D., Brock-Utne, J.G. and Gaffin, S.L. (1988) Circ. Shock 25, 223–230.
[14] Bouchama, A., Parhar, R.S., El-Yazigi, Sheth, A.K. and Al-Sedairy, S. (1991) J. Appl. Physiol. 70, 2640–2644.
[15] Buettner, G.R. (1987) Free Radic. Biol. Med. 3, 259–303.
[16] Finkelstein, E., Rosen, G.M. and Rauckman, E.J. (1982) Mol. Pharmacol. 21, 262–265.
[17] Finkelstein, E., Rosen, G.M. and Rauckman, E.J. (1980) J. Am. Chem. Soc. 102, 4994–4999.
[18] Samuni, A., Carmichael, A.J., Russo, A., Mitchell, J.B. and Riesz, P. (1986) Proc. Natl. Acad. Sci. USA 83, 7593–7597.
[19] Thor, H., Smith, M.T., Hatzell, P., Bellomo, G., Jewell, S.A. and Orrenius, S. (1982) J. Biol. Chem. 257, 12419–12425.
[20] Hall, D.M., Buettner, G.R., Matthes, R.D. and Gisolfi, C.V. (1994) J. Appl. Physiol. 77, 548–553.