0014-4754/94/050465-04$1.50 + 0.20

Effects of chronic caffeine feeding on the activities of oxygen free radical defense enzymes in the growing rat heart and liver

M. J. Rossowska and T. Nakamoto

Laboratory of Perinatal Nutrition and Metabolism, Department of Physiology, Louisiana State University Medical Center, 1100 Florida Avenue, New Orleans (Louisiana 70119 USA)
Received 11 August 1993; accepted 11 February 1994

Abstract. The purpose of the present study was to determine the relationship between concentration of Zn, Cu and Fe, and the catalase, glutathione peroxidase and superoxide dismutase activities in the heart and liver of newborn rats whose dams were fed a diet supplemented with caffeine. Heart Zn levels of the 22- and 30-day-old rats of the caffeine group showed a decrease, whereas liver Zn levels showed an increase compared to the control. Cu levels in the liver at day 22 in the caffeine group were less than in the control. Cu- and Zn-containing superoxide dismutase activities showed an increase in the hearts of the caffeine group compared to the control. The activity of catalase and glutathion peroxidase showed no difference in the heart and liver between the groups. The present study suggests the possible involvement of superoxide dismutase enzyme in the impairment of heart formation as a result of chronic caffeine intake in the early growing period.
Key words. Caffeine; trace elements; antioxidant enzymes; liver and heart; neonate.

Chronic caffeine consumption during early growth and development impairs cardiac performance in rats. Using an isolated heart preparation, the hearts of caffeine-fed rats over a period of 88 days after birth have shown a significant reduction of cardiac output, stroke volume, mean aortic pressure and estimated myocardial work when compared with the non-caffeine control group[1]. Even when the caffeine feeding period is shortened to 50 days after birth, various factors measured have indicated similar impairment of function[2]. Chronic caffeine ingestion exacerbates renovascular hypertension and results in a moderate to severe myocardial fibrosis[3]. The mechanism which produces permanently altered cardiovascular function in the growing heart as a result of chronic caffeine feeding is not known at the present time.

Exposure to caffeine decreases the level of zinc in the heart but has not affected Zn absorption[4]. It has been proposed that among the biological functions of zinc are protection against free radical damage[5]. Zinc competes with copper for binding to biological molecules[6]. Alterations in iron metabolism may also be mediated in a zinc-copper interaction[7,8]. If so, then expression of antioxidant metalloenzyme activities in developing tissues may be limited by the availability of their trace metal cofactors in which caffeine intake may directly be associated. The enzymes of the primary antioxidant defense system which protect against reactive oxygen species are: Cu, Zn superoxide dismutase (Cu, Zn SOD), catalase and glutathione peroxidase (GSH-Px). These enzymes catalyze the distribution of O_2^- (Cu, Zn SOD) and H_2O_2 (catalase and GSH-Px). The objective of this study is to characterize the chronic effect of caffeine intake on oxygen free radical defense enzyme activities (Cu, Zn SOD, catalase, GSH-Px) in the growing heart. We have also measured these effects on the liver to compare possible different effects of caffeine on this organ in growing animals.

Materials and methods

Timed-pregnant Sprague-Dawley rats were purchased from the breeder (Holtzman Strain, Madison, Wisconsin, USA) and fed Purina Laboratory Chow until delivery. At birth, 8 randomly selected pups were assigned to each dam. Dams with pups were then randomly divided into two groups. Group 1 was fed a 20% protein diet as a control[9]. Group 2 was fed a 20% protein diet supplemented with caffeine (20 mg/kg b. wt of dams). When the offsprings were weaned at day 22, some of them were killed by decapitation. The hearts and livers were removed, blotted, weighed and immediately frozen in liquid nitrogen. Tissues were stored at −80 °C prior to all analyses. Blood samples were collected in heparinized tubes. All experiments were conducted between 10.00 and 12.00 hours. Randomly selected pups from each dam of the control group were fed continuously on the same 20% protein diet. Pups from the experimental group of dams were fed a 20% protein diet supplemented with caffeine (20 mg/kg b. wt of pups). Caffeine levels in the diet were adjusted for increased b. wt and food intake as described[10].

Fresh diet was made every week. All pups were housed individually and water was provided ad libitum. On day 30 pups were killed by decapitation and samples were

collected in the same way as samples of the 22 day old pups. The tissues were homogenized with double distilled deionized water to determine caffeine levels and various mineral contents, as described previously[11], using atomic absorption spectrophotometry (Model 2380, Perkin-Elmer Co.). Analyses were validated using National Institute of Standards and Technology (Gaithersburg, Maryland, USA) standard bovine liver (1577b). Values for Cu, Zn, Fe agreed within 97–99% of the stated standard value. The caffeine concentration of tissues samples were measured by the method described previously[12].

For determination of enzyme activities, tissues were homogenized using polytron homogenizer in various buffers according to the assay procedure for SOD, catalase and GSH-Px. The homogenate was centrifuged at 6000 *g* for 15 min and supernatants were used in the enzyme assays (total SOD, GSH-Px, catalase) protein determination. Superoxide dismutase [EC1.15.1.1] activity was determined according to the method of Bauchamp and Friovich[13] modified by Oberley and Spitz[14]. To assay Zn, Cu SOD activities the supernatant was treated with 0.4 volumes of ethanol:chloroform (25:15) followed by centrifugation at 6000 *g* for 20 min[15]. The supernatant obtained was used for the assay of Cu, Zn SOD activity.

The assay is based on the inhibition of the conversion of nitro-blue tetrazolium (NBT) to blue formazon by SOD, mediated by superoxide radical generated by the xanthine oxidase system. The reaction of NBT was followed at 560 nm with a Hitachi U-2000 spectrophotometer. One unit is equal to the amount of protein which gives half-maximum inhibition. Xanthine oxidase inhibitory or stimulatory activity was measured in a representative sample from each tissue by following the conversion of xanthine to uric acid at 290 nm. No significant xanthine oxidase inhibitory or stimulatory activity was seen in any tissue. Glutathione peroxidase [EC 1.11.1.9] activity was measured using tetra-butyl hydroperoxide as substrate[16]. Units of GSH-Px were expressed as micromoles NADPH oxidized per minute.

For determination of catalase [EC 1.11.1.6] activity, the supernatants (6000g) were treated with ethanol and Triton X-100 prior to analysis to give the full expression of catalase activity[17]. Catalase activity was measured as decomposition of hydrogen peroxide. Hydrogen peroxide was measured by reacting it with standard excess of $KMnO_4$ and measuring the residual $KMnO_4$ spectrophotometrically. Catalase activity was expressed as k/mg protein, where k is the first-order reaction rate constant[17,18]. Protein was determined by the Lowry method[19] using bovine serum albumin as a standard. Statistical analyses were performed using the Student t-test with 5% considered significant.

Results

The activity of Cu, Zn SOD in the hearts of the caffeine group was significantly higher than in the non-caffeine controls in both 22- and 30-day-old rats. The activities of the total SOD, catalase and GSH-Px showed no differences between the caffeine and non-caffeine groups in either 22- or 30-day old rats (table 1). Higher catalase and GSH-Px activities were observed in 30-day-old rats than in 22-day-old rats.

The activities of the total SOD, Cu, Zn SOD, catalase and GSH-Px in the liver showed no difference between caffeine and non-caffeine control groups in both 22-and 30-day-old rats, but these activities in 30-day-old rats were much higher than in 22-day-old rats (table 1).

Zinc concentration in the hearts of the caffeine group was less than in the non-caffeine controls in both 22- and 30-day-old rats. Copper and iron concentrations showed no differences between the groups in both 22- and 30-day-old rats (table 2).

Zinc concentration in the liver in the caffeine group was significantly higher than the non-caffeine controls at 22 days, whereas Cu concentration in the caffeine group was less than in the non-caffeine group. Copper concentration in the livers of 30-day-old rats showed much lower levels than that of 22-day-old rats, and no difference between caffeine and non-caffeine groups existed at day 30. Iron concentration in the livers of 30-day-old rats was higher than in 22-day-old rats, but showed no difference between caffeine and non-caffeine groups in both 22- and 30-day-old rats (table 2). Caffeine concentration in the livers and hearts of 30-day-old rats showed higher levels than in 22-day-old rats (table 1).

Discussion

The effects of chronic caffeine intake on antioxidant metalloenzyme cofactors Zn, Cu, and Fe and their enzyme activities were investigated in the present experiments. If the present dosage of caffeine is translated into human consumption, it is equivalent to a daily intake of 2–3 cups of coffee in lactating women or growing offspring after weaning. This is due to the shorter half-life of caffeine in rats compared to humans[20]. This corresponds well to the normal range of daily intake of coffee among child-bearing women[21]. We have also calculated caffeine intake of pups through the maternal milk based upon the milk collected at days 15 and 22. Considering the pups' intake of nibbled maternal food between days 15 and 22, intake of caffeine by pups during the entire lactational period corresponds to a range of less than 1/2 cup of coffee to slightly more than one cup of coffee.

In this study caffeine significantly decreased the zinc levels in the heart in both 22- and 30-day-old rats. On the other hand, in the liver caffeine decreased Cu and increased Zn concentrations in 22-day-old rats. It is

Table 1. Effect of chronic caffeine feeding on superoxide dismutase, catalase, glutathione peroxidase, and caffeine concentration in the heart and liver of 22- and 30-day-old rats

		22-Day-Old		30-Day-Old	
		Control	Caffeine	Control	Caffeine
Total SOD (U/mg p)	Heart	196.09 ± 8.70[a] (4)	221.22 ± 15.75 (4)	191.84 ± 6.45 (5)	225.00 ± 15.51 (5)
	Liver	304.83 ± 21.89 (5)	318.64 ± 20.46 (5)	381.95 ± 13.47** (5)	324.57 ± 26.60 (5)
CuZnSOD (U/mg p)	Heart	122.27 ± 9.10 (6)	156.36 ± 8.03* (8)	133.89 ± 11.99 (7)	190.17 ± 5.81* (5)
	Liver	199.51 ± 14.52 (5)	187.39 ± 14.18 (5)	272.47 ± 16.65** (5)	247.30 ± 32.20 (5)
Catalase (K/mg p)	Heart	0.327 ± 0.010 (3)	0.330 ± 0.030 (3)	0.461 ± 0.019** (3)	0.451 ± 0.010 (3)
	Liver	6.96 ± 0.45 (7)	7.70 ± 0.99 (7)	14.38 ± 1.46** (6)	14.19 ± 1.28 (6)
GSH-Px (μmol/min/mg p)	Heart	0.174 ± 0.022 (5)	0.143 ± 0.014 (5)	0.220 ± 0.019 (5)	0.260 ± 0.010 (5)
	Liver	0.418 ± 0.007 (4)	0.393 ± 0.032 (4)	0.516 ± 0.029** (5)	0.526 ± 0.010 (5)
Caffeine (nmol/100 mg p)	Heart		1.15 ± 0.29 (4)		2.29 ± 0.55 (5)
	Liver		0.54 ± 0.17 (5)		1.29 ± 0.55 (5)

[a]Each value represents the mean ± SE; number of samples is given in parenthesis.
*Significantly different from control values (Student's t-test); $p < 0.05$.
**Significantly different from non-caffeine control values of the 22-day-old rats; $p < 0.05$.

Table 2. Effect of caffeine on zinc, copper and iron concentrations in the heart and liver of 22-day and 30-day-old rats (μg/g wt)

		22-Day-Old		30-Day-Old	
		Control	Caffeine	Control	Caffeine
Zn	Heart	18.02 ± 0.16[a] (6)	16.30 ± 0.38* (6)	17.59 ± 0.44 (6)	15.58 ± 0.28* (6)
	Liver	24.11 ± 0.56 (9)	27.86 ± 1.12* (7)	30.20 ± 2.01** (8)	26.57 ± 1.33 (9)
Cu	Heart	4.23 ± 0.16 (4)	4.58 ± 0.18 (4)	4.87 ± 0.28 (6)	5.45 ± 0.16 (5)
	Liver	19.72 ± 1.82 (7)	13.90 ± 1.13* (5)	9.33 ± 0.94** (10)	8.74 ± 0.61 (7)
Fe	Heart	57.99 ± 4.23 (4)	50.06 ± 1.96 (4)	56.78 ± 1.55 (4)	53.04 ± 1.39 (4)
	Liver	26.70 ± 2.09 (9)	24.01 ± 1.43 (7)	63.21 ± 2.89** (8)	54.12 ± 5.27 (9)

[a]Each value represents the mean ± SE; number of samples determined is given in parenthesis.
*Significantly different from control values; $p < 0.05$.
**Significantly different from non-caffeine control values of 22-day-old rats; $p < 0.02$.

evident that these metals are essential for maturational processes, since deficiencies in their supply lead to several structural and functional abnormalities[22,23]. In the non-caffeine control, Cu concentration in liver decreased whereas Fe increased after the third week postbirth. Liver Zn concentration did not change dramatically compared to Cu and Fe levels during the time study. Cu, Zn and Fe in the hearts have shown no change during the same time period. Similar results have been demonstrated previously in rat and mice tissues[24,25]. The reason for this increase in liver iron concentration is not clear. It is possible that the pups begin to consume maternal food around the end of the 2nd week so that dietary intake of iron by pups is consderably increased after the third week. This possibly affected the Fe concentration in the liver, but not in the heart. The hepatic catalase activity changed in parallel with Fe concentration. Catalase activity in the liver is modulated by availability of its metal cofactor, Fe[26]. The decline of liver Cu concentration with age is probably due to Cu mobilization and tissue growth[27].

A variety of studies have suggested that there is increased free radical production in tissues or isolated membranes from Zn-deficient and Cu-deficient ani-

mals[28–30]. This may explain why the increased Cu, Zn SOD activity was observed in the decreased Zn levels in the heart, although it has also been shown in a nutritional study that Zn deficiency has little effect on the Cu, Zn SOD activity[31].

Cu, Zn SOD activity has been considered a good marker of copper status[30,31]. However, in the present study, liver Cu, Zn SOD activity did not correlate with the tissue level of Cu concentration during the neonatal period. It is possible that there may be factors other than availability of metal cofactors which determine the expression of Cu, Zn SOD in the rat liver during this period.

Tissue Cu levels were decreased in the liver at day 22 after caffeine. However, Cu, Zn SOD activity did not change. Although this enzyme could be influenced by the decreased liver Cu concentration, which decreases this enzyme activity, caffeine could have increased its activity at this time of the development stage. If so, then this may explain why Cu, Zn SOD activity in hearts increased, whereas there was no change in Cu, Zn SOD activity in livers in spite of decreased Cu tissue concentration.

In other data it is suggested that divalent metals other than Cu could also coordinate the expression of Cu, Zn SOD activity[32]. On the other hand, fetal lung cells have shown elevated Cu, Zn SOD activity in response to dexamethasome[33]. It is possible that hormonal influence, aging and cellular differentiation may also have a role in Cu, Zn SOD regulation. The dismutation of O_2^- by SOD generated H_2O_2, which itself is a dangerous oxidant in cells. Thus, to respond to an increase in O_2^- cells must not only elevate Cu, Zn SOD, but also catalase and GSH-Px as well, which regulate H_2O_2.

Hydrogen peroxide is hazardous to mitochondria and other cell components. Consequences of elevated mitochondrial H_2O_2 concentration are lipid peroxidation, disruption of calcium homeostasis, inactivation of respiratory chain carriers and other mitochondrial enzymes[34–36]. Lipid peroxidation of biological membranes occurring secondary to excess H_2O_2 production can lead to cell injury[37]. Our preliminary results demonstrate some changes in the structure of heart mitochondria after caffeine feeding (data not shown). On the other hand, it has been reported that H_2O_2 is poorly reactive with heart mitochondria, having little influence on electron transport and transmembrane potential[36,38]. Clearly, additional work is necessary to elucidate the effect of chronic caffeine intake on the growing heart. The observed increase of Cu, ZnSOD activity after chronic caffeine intake by pups during critical time periods of growth may be a contributing factor to the pathogenesis of the heart, which has previously been observed in the form of various impaired functions[1,2].

* To whom reprint requests and correspondence should be addressed.

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