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High environmental temperature potentiated marker of oxidative cellular damage and renal expression of p38 MAPK in male rats fed a high salt diet

Francis Muyiwa Agbaraolorunpo · Ahmed Kolade Oloyo · Adesina Paul Arikawe · Chikodi Nnanyelu Anigbogu · Olusoga Adekunle Sofola

Department of Physiology, Faculty of Basic Medical Science, College of Medicine of the University of Lagos, Idiaraba, Lagos, Nigeria

ABSTRACT

Aim: Oxidative stress, heat shock protein (HSP70) and p38 mitogen-activated protein kinase (MAPK) are important functional cellular signals involved in the pathophysiology of cardiovascular diseases. This study investigated the effect of high environmental temperature (HET) and high salt diet (HSD) respectively and together on systemic oxidative stress, nitric oxide (NO), HSP70 and renal p38 MAPK.

Methods: Thirty-two male Sprague-Dawley rats were divided into four groups with eight rats in each group: Control rats (C) fed a normal diet; salt-loaded rats (S) fed a HSD (8%NaCl); normal diet rats (H) exposed to HET (38.5 ± 0.5°C 4h daily for 8weeks); and salt-loaded rats (SH), fed a HSD and exposed to HET. Circulatory oxidative stress parameters (SOD: superoxide dismutase; GSH: glutathione; CAT: catalase; MDA: malondialdehyde), HSP70 and renal p38 MAPK were determined by colorimetric, enzyme-linked immunosorbent assay (ELISA) methods and immunohistochemistry (IHC) techniques respectively.

Results: Plasma GSH concentration and CAT activity decreased significantly, with significant increase in MDA concentration in all the rat groups compared to control. However, MDA in SH rats was significantly higher than in either S or H rats. Circulatory HSP70 and NO were significantly raised in S and H rats but unchanged in SH rats compared to control. Conversely, renal expression of p38 MAPK was significantly increased in H and SH rats compared to control, but SH rats had significantly higher level than either S or H rats. SH rats also had weight gain slowing compared to control.

Conclusion: Our findings indicate that prolonged exposure to HET and HSD intakes synergistically increased renal p38 MAPK and circulatory product of oxidative cellular damage without alteration in circulatory HSP70 and NO.

Keywords: HSP70, p38 MAPK, high environmental temperature, high salt diet, oxidative stress.

✉ *Dr. Francis Muyiwa Agbaraolorunpo*
Department of Physiology, Faculty of Basic Medical Science, College of Medicine of the University of Lagos, Idiaraba, Lagos, Nigeria

Introduction

Exposure to high environmental temperature is on the rise worldwide due to global warming [1]. Dietary high salt intake is also rising (>5 grams per day) in many countries around the globe and was reported to be responsible for 3 million deaths from cardiovascular related causes [2]. High environmental temperature has also been suggested as a cardiovascular risk factor [3]. Sadly, cardiovascular disease (CVD) is identified as the leading cause of death and disability worldwide [4]. Our previous work demonstrated that high environmental temperature interacted with high salt diet in experimental rats to exacerbate salt-induced increase in blood pressure and myocardial workload [5]. Oxidative stress has been identified as the main cause of increased mortality in cardiovascular disease [6], with p38 mitogen-activated protein kinase (MAPK) and heat shock protein (HSP) playing an important regulatory roles.

HSP is induced by a wide variety of stimuli including pressure or volume overload, thermal stress, salt-induced stress [7]. HSPs exhibit cytoprotective effects on different organs, and these may include the correction of folding of regulatory proteins, degradation of abnormal protein, protection of cytoskeleton as well as inhibition of apoptosis [8]. HSP70 also enhances nitric oxide production [9]. So far, the presence of HSP70 has been demonstrated in several experimental models of hypertension including salt sensitive hypertension [7].

p38 MAPK is an example of intracellular protein kinase involved in cellular signaling and activated by numerous extracellular stimuli [10]. When activated, p38 MAPK is reported to cause DNA damage and cell death [11]. Its activation has also been shown to upregulate specific inflammatory cytokines in several biological contexts including kidney damage [12], myocardial injury and spontaneous hypertension in stroke prone rats [13]. This current study is aimed at investigating the plausible role of oxidative stress, HSP70 and alpha p38 , otherwise referred to as p38 MAPK, in the earlier reported synergistic interaction between high environmental temperature and high salt diet on cardiovascular function [5].

Materials and Methods

Experimental animals

The study protocol was approved by the Ethics committee of College of Medicine of the University of Lagos (CMUL/HREC/11/18/471) and was performed at the Department of Physiology Research Lab of the College of Medicine, University of Lagos. Animal care and handlings were done according to the National Research Council (US) Committee for the Care and Use of Laboratory [14]. Thirty-two male Sprague-Dawley rats weighing between 95 and 110g were used for the study. The rats were maintained on a 12h dark/light cycle at $25 \pm 0.5^{o}C$ room temperature in the animal house and were allowed access to standard rat chow and clean tap water ad libitum throughout the study. The rats were randomly divided into one of the following experimental groups with 8 rats per group.

Group 1: Control rats (C), were fed with normal diet containing 0.3% of NaCl and exposed to room temperature of $25 \pm 0.5^{o}C$ throughout the 8 weeks of the experiment.

Group 2: Salt-loaded rats (S), were fed with high salt diet containing 8%NaCl and exposed to room temperature of $25 \pm 0.5^{o}C$ throughout the 8 weeks of the experiment.

Group 3: Heat-exposed rats (H), were fed on normal diet but exposed to high environmental temperature at $38.5 \pm 0.5^{o}C$ (relative humidity between 65 and 75%) 4 hours daily for 8 weeks.

Group 4: Salt-loaded + Heat-exposed rats (SH), were fed with high salt diet (containing 8%NaCl) and exposed to high environmental temperature at 38.5±0.5°C (relative humidity between 65 and 75%) 4 hours daily for 8 weeks.

Experimental protocol

Salt-loaded rats were fed with high salt diet as described by Sofola et al., [15] for 8 weeks. Heat- exposed rats were acclimatized to HET for one week starting from 30°C to 35°C with a daily temperature increase of 1°C. Thereafter, the animals were exposed to HET using the method described by Barney & Kuhrt [16], but with a slight modification of the temperature to a higher level of 38.5±0.5°C and a relative humidity between 65 and 75%. The consideration was due to the relatively high room temperature of our environment located in the South West Nigeria of Sub-Saharan Africa in the tropics. Heat exposure took place for 4 hours daily for 6 (days/week) for 8 weeks in environmental chambers from 9am to 1pm. Environmental temperature was monitored with environmental thermometer (HTC-2 OEM, Zhejiang, China).

Determination of weekly body weight gain

Percentage body weight gain was determined on weekly basis in all the experimental rats (n=8) with digital weighing scale using the following formula: $\frac{Wc - Wp}{Wp}$ *100; *Wc*, current weight; *Wp*, previous weight.

Animal sacrifice

The rats were anaesthetized on the 8-week with ketamine (75mg/Kg) intraperitoneally, blood was collected via cardiac puncture into heparinized bottles and centrifuged at 3000rpm for 15 minutes to extract plasma samples, which were stored at -25°C until further analysis for super oxide dismutase (SOD) and catalase

(CAT) activities, reduced glutathione **(GSH)**, malondialdehyde (MDA) and nitric oxide (NO) concentrations. The rats were perfused through the ascending aorta with 40 ml of 0.01 M phosphate buffered saline (PBS, pH 7.4) containing Heparin (5 IU/mL) at room temperature. This was followed by 400 mL of 4% paraformaldehyde (PFA) in phosphate buffer (PB) 0.1 M sodium (4% PFA, pH 7.4) at 4 ° C. The kidneys were carefully isolated and removed immediately after perfusion, post-fixed in 4% PFA for 2 hours at 4 ° C and finally stored at room temperature in 10% formal saline until p38 MAPK study using immunohistochemistry (IHC) technique.

Super oxide dismutase activity

Super oxide dismutase activity **(**SOD) activity was determined by the method of Misra and Fridovich [17] based on the principle that superoxide anion ($\bullet O_2^-$) causes the oxidation of epinephrine to adrenochrome, a transition that is inhibited by SOD (enzymes) in the biological sample. Briefly, 14.3g of Na2CO3.10H$_2$O (Sigma Chemicals Ltd, USA) and 4.2g of NaHCO3 (Sigma Chemicals Ltd, USA) were dissolved in distilled water and made up to 1000ml mark in a liter standard flask. Then the solution was adjusted to pH 10.2. Next, 0.0013g of epinephrine (molecular weight of 182.21gmol-1) (Sigma Chemicals Ltd, USA) was dissolved in 250ml of distilled water. Thereafter, 0.1ml of plasma was diluted in 0.9ml of distilled water to make a 1 in 10 dilution. An aliquot of 0.2ml of the diluted plasma sample was added to 2.5ml of 0.05M carbonate buffer (pH 10.2) to equilibrate, and the reaction was started by adding 0.3ml of freshly prepared 0.3mM epinephrine to the mixture which was quickly mixed by inversion. The increase in absorbance at 480nm was monitored every 30seconds for 150 seconds in

spectrophotometers (T70UV/VIS, UK). 1 unit of SOD activity was regarded as the amount of SOD required to cause 50% inhibition of the oxidation of epinephrine to adrenochrome during 1 minute.

Catalase activity

Catalase (CAT) activity was evaluated colorimetrically according to the method of Sinha [18] by determining the rate of consumption of H_2O_2 (hydrogen peroxide) at absorbance measured with a spectrophotometer at 570nm. This method is based on the fact that dichromate in acetic acid is reduced to chromic acetate when heated in the presence of H_2O_2 with the formation of perchromic. Briefly, different amounts of H_2O_2 ranging from 20 to 160 mmoles were taken in small test tubes and 2ml of dichromate/acetic acid was added to each. This immediately produces an unstable blue precipitate of perchromic acid. Following the heating of the solution for 10mins in boiling water the color of the solution changed to stable green due to the formation of chromic acetate. After cooling at room temperature, the volume of the reaction mixture was made to 3ml with distilled water and the absorbance was measured with a spectrophotometer at 570nm. Next, 0.1ml of plasma was mixed with 4.9ml of distilled water to give a 1 in 5 dilution of the sample. 1ml of the diluted plasma preparation (test sample) was then rapidly mixed with the reaction mixture by gentle spinning motion at room temperature. Thereafter, 1ml of the reaction mixture was withdrawn and blown into a test tube containing 2ml of dichromate/acetic acid reagent at 60 seconds intervals for 3minutes. The H_2O_2 contents of the withdrawn sample were determined. The decomposition of H_2O_2 by catalase was determined by using the equation for a first – order reaction. $K = 1/t \log S_o/S$. Where, S_o is the initial concentration of

H_2O_2. S is the concentration of peroxide at t min (60 seconds interval). T is time – interval (1minute).

Reduced glutathione

Reduced glutathione (GSH) was determined by the method described by Habing et al [19]. Briefly, 0.2ml of plasma sample was mixed with 1.8ml of distilled water to give 1 in 10 dilutions. About 3ml of precipitating reagent (4% sulphosalicyclic acid) was added to the diluted plasma sample and then allowed to stand for 10minutes form precipitate to occur. 0.5ml of supernatant was withdrawn and added to 4ml of phosphate buffer followed by 0.5ml of Ellman's reagent. The blank was prepared with 4ml of 0.1M phosphate buffer pH 7.4, 1ml of diluted precipitating solution and 0.5ml of Ellman's reagent (DTNB). The absorbance was read within 20minutes of color development at 412nm against blank using spectrophotometer. Reduced glutathione concentration was proportional to the absorbance at 412 nm.

Malondialdehyde

Malondialdehyde (MDA) resulting from lipid peroxidation of biological membrane was estimated with the method described by Mihara and Uchyama (1978)[20]. This is based on the formation of pink colour complex when MDA interact with thiobarbituric acid (TBA) at 535nm. Briefly, Trichloroacetic acid (TCA; 15%), hydrochloric acid (HCL: 0.24N) and thiobarbituric acid (TBA; 0.37%) were prepared and mixed in ratio 1:1:1. Thereafter, 1ml of filtrate from centrifuged plasma was added into the 2ml of the acidic solution. The absorbance was read at 535nm in spectrophotometers.

Plasma nitric oxide measurement

Total plasma concentration of nitrites (NO_2^-) and nitrate (NO_3^-) were used as an indicator of

plasma nitric oxide (NO). Nitrites (NO_2^-) using the Griess reaction [21]. Nitrate and nitrite were measured using the cadmium reduction and diazotization techniques respectively. Concentrations of nitrate and nitrite were determined calorimetrically by reading absorbance with UV-Spectrophotometer, (CAMSPEC 309, and USA) at 500 nm and 507 nm respectively. Briefly, 1ml each of plasma was measured in a volumetric flask with 6 ml of deionized water. In the mixture, 1ml of 5% mercuric chloride was added , made up to 10 ml and filtered into a 25ml standard volumetric flask .The solution was made up to mark with deionized water. A standard preparation and a blank control were also prepared using the same method adopted in preparing the samples except that no plasma was added. 10 ml of each solution were measured in 20 ml test tubes before adding nitrate and nitrite powder pillow reagents (HACH Company, USA) respectively in each. This was then allowed to stand for 20 minutes for a color development and the readings were taken respectively.

Heat Shock Protein 70 (HSP70)

Plasma HSP70 concentrations was determined using rat HSP70 elisa kit (MyBiosource, San Diego, USA) according to the manufacturer instruction. Briefly, 0.1ml of plasma sample and 0.1 ml of graded concentration of standard preparation respectively were added into micro wells pre-coated with HSP70 antibody and incubated for 90minutes at 37°C, followed by the addition of 0.1ml of Biotin-label antibody and incubation for 60 minutes at 37°C. The micro wells were washed with buffer water thrice. 0.1ml of SABC solution each was added into each of the well and incubated for 30minutes at 37°C followed by washes five times. Then, TMB substrate (90μL) was added into the wells in dark within 15 minutes. A stop

solution (50μL) was then added into each of well to stop the reaction. The absorbance was read at 450nm in microplate immediately and used to determine the concentration of HSP70.

Determination of renal expression of alpha p38 MAPK by immunohistochemistry techniques

Renal expression of p38 MAPK was determined using Rat IHC-P kit (Cell Signaling Technology, USA) according to the manufacturer instruction briefly described below. *Kidney section and deparaffinization*: Kidneys were processed into blocks with liquid paraffin. The blocks were sectioned with microtomes and placed on charge slides. This was followed by the deparaffinization and hydration of the kidney sections by incubating the sections in three washes of xylene for 5 min each, followed by incubation in two washes of 100% ethanol for 10min each and another two washes in 95% ethanol for 10 mins each. Thereafter sections were washed twice in dH2O for 5 min each. *Antigen unmasking*: Slides were heated in a microwave submersed in 1X citrate unmasking solution until boiling is initiated, followed with 10 min at sub-boiling temperature (95-98°C).The slides were then cooled on bench top for 30 min. *Staining:* Sections were washed in dH2O three times for 5 min each. The sections were then incubated in 3% hydrogen peroxide for 10 min. This was followed by wash in dH_2O two times for 5 min each, followed by a wash in buffer for 5 min each. Next, the sections were blocked with 100-400μl of blocking solution (normal goat serum blocking solution) for 1 hr at room temperature. The blocking solution was removed from the sections and 100-400μl of primary antibody diluted in Signal stain Antibody Diluent was added to each section, followed by an incubation overnight at 4°C.The primary

antibody solution was removed by washing sections with wash buffer three times for 5 min each. Then each section was covered with 1-3 drops Signal Stains Boost Detection Reagent (HRP) and incubated in a humidified chamber for 30 min at room temperature. The Signal Stain Boost Detection Reagent (HRP, Rabbit) was equilibrated to room temperature before use. Next, section was washed three times with wash buffer for 5 min each. One drop (30μl) of SignalStain DAB Chromogen Concentrate was added to 1 ml SignalStain DAB Diluent and mix well before use.100-400μl Signal Stain DAB was applied to each section 5-10 min followed by immersion of sections in dH2O and subsequent sections counterstaining with hematoxylin. The sections were washed in dH2O two times for 5 min each, followed by dehydration of section. ***Dehydrate sections:*** Sections were incubated in 95% ethanol two times for 10 seconds each, repeated in 100% ethanol, followed by the incubation of sections two times for 10 secs. This was then repeated in xylene and incubated two times for 10 each section. Finally, sections were mounted with coverslips and mounting medium. Histoscore for positive stains was evaluated as following: (1 × % weakly stained cells) + (2 × % moderately stained cells) + (3 × % strongly stained cells) [22].

Statistical analysis: Data were presented as Mean ± SEM. Differences in experimental rat groups were compared with One-way ANOVA followed by Tukey post-hoc test, Pre and post-rectal temperatures were compared with Paired t-test. *P<0.05* was regarded as statistically significant .Graph pad 5 software package (USA) was used for the analysis.

Results

Oxidative stress parameters (SOD, CAT, GSH and MDA) of salt-loaded rats exposed to high environmental temperature

Plasma CAT activity and GSH concentration were lower in rats fed a high salt diet alone *(p<0.05, p<0.05)* and in rats exposed to high environmental temperature alone *(p<0.05, p<0.05)* compared to control rats. In addition, plasma MDA concentration was higher in both experimental rats compared to control *(p<0.01, p<0.001)*. Similarly, plasma CAT activity and GSH concentration were lower in rats exposed to the combined environmental factors *(p<0.05, p<0.01)*, with elevated plasma MDA concentration *(p<0.001)* compared to control rats. However, plasma MDA concentration was higher in rats exposed to the combined environmental factors *(p<0.01)* than in rats exposed to high either environmental temperature alone or in rats fed a high salt diet alone. This indicates a synergistic effect of the two environmental factors on circulatory MDA.

**Table 1.** Effect of HET on oxidative stress parameters in salt-loaded rats**.**

Groups	SOD (U/mg of protein)	CAT (U/mg of protein)	GSH (μmol/ml)	MDA (∞mol/ml)
Control	945.4 ± 106	467.7 ± 15.9	17.0 ± 1.1	3.0±0.5
Salt	526.5 ± 54.3	239.1 ± 40.6*	10.6 ± 0.3*	7.3±0.5**
Heat	502.7 ± 97.6	260.3 ± 55.0*	10.7 ± 0.9*	13.5±0.5***
Salt+heat	553.7 ± 61.7	253.5 ± 61.4*	9.2 ± 1.4**	18.9±1.3***###

*SOD: super oxide dismutase; CAT: catalase; GSH: reduce glutathione; MDA: Malondialdehyde; *p<0.05, **p<0.01 ***p<0.001 vs Control, ###p<0.001 vs Salt, ###p<0.001 vs Heat presented as Mean ± SEM (n=8). HET: high environmental temperature.*

Meanwhile, plasma SOD activity although tended towards a decline in all the experimental rats was not significantly different compared to control rats (Table 1). The decreased plasma CAT activity, GSH concentration and increased MDA above are indicative of increased oxidative stress in all the experimental rats.

p38 MAPK expression in the kidney of salt-loaded rats exposed to high environmental temperature

p38MAPK was weakly expressed in the kidneys of control rats (A) and in rats fed on high salt diet alone (B), but was moderately expressed in the kidneys of rats exposed to HET
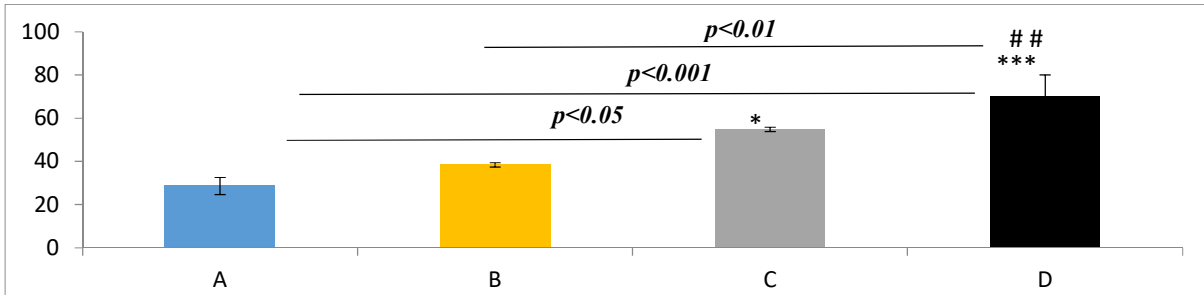
**Figure 1a. Percentsge p38 MAPK expression in renal tubules.**
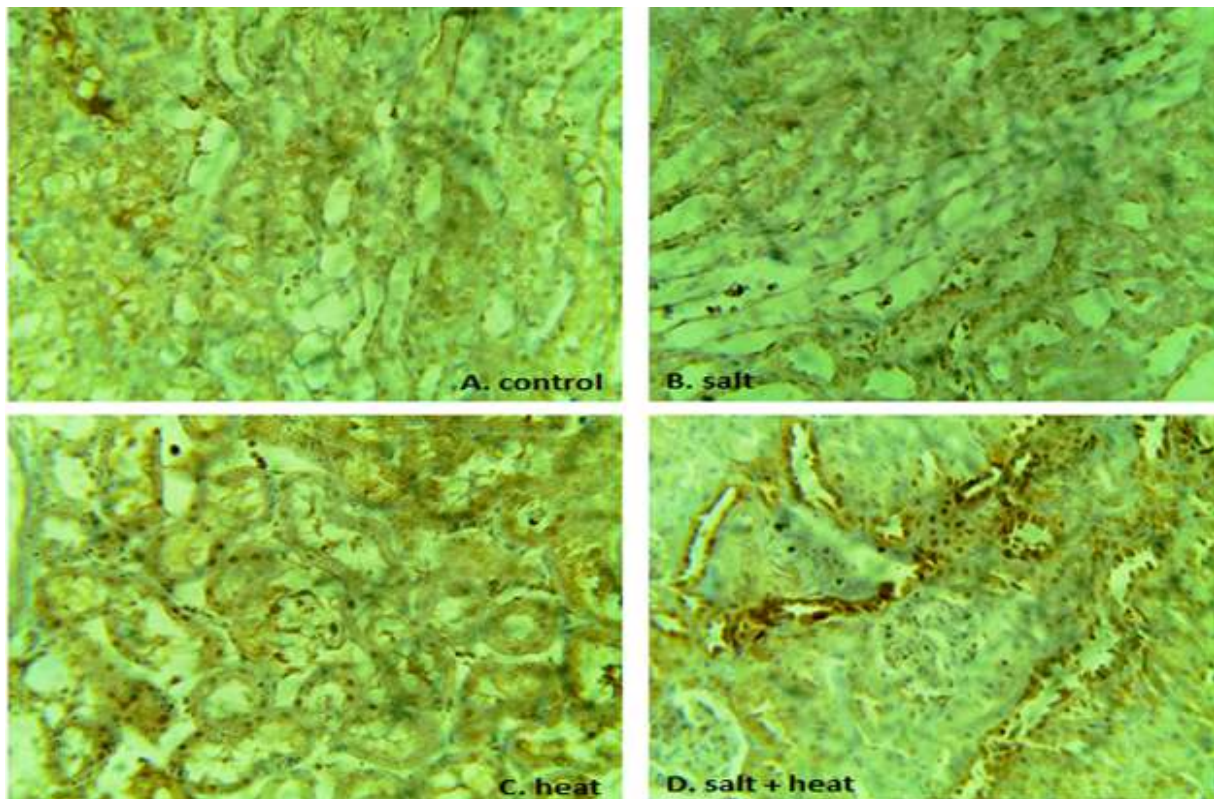
Figure 1b. Immunohistochemistry of renal tubules showing the expression of p38 MAPK in the kidneys of experimental rats: control rats (A) were fed with normal diet (0.3% NaCl), salt-loaded rat (B) were fed with high salt diet (% NaCl), heat-exposed rats were fed with normal diet but exposed to HET (C), salt-loaded + heat-exposed rats (D) were fed with high salt diet (8%NaCl & exposed to HET. The expression of p38 higher in salt + heat rats was higher than the expression in salt-loaded rats (B) as well as in the heat-exposed rats (C). (Magnification, X 400). Formalin-fixed and paraffin embedded rat kidneys labeled with Anti-p38 MAPK Polyclonal Antibody, Unconjugated (Cell Signaling Technology, USA) at 1:200 followed by conjugation to the secondary antibody and DAB staining. Data presented as mean ± SEM (n=5).

alone (C) *(p < 0.05)* and strongly expressed in the kidneys of rats exposed to combined environmental factors (D) compared to control *(p < 0.001)*. Furthermore, the expression of p38 MAPK in the kidneys of rats exposed to the combined environmental factor was stronger *(p < 0.01)* than those of the salt group, indicating a possible synergistic interaction between HSD and HET on p38 MAPK expression (Figure 1a & b).

Circulatory HSP70 and nitric oxide in salt-loaded rats exposed to high environmental temperature

In comparison with control, plasma HSP70 (ng/ml) was significantly higher in rats fed a high salt diet alone *(1.1 ± 0.1 vs 1.6 ± 0.2, P < 0.01)* and in rats exposed to high environmental temperature alone *(1.1 ± 0.1 vs 1.5 ± 0.1, P < 0.05)*, but unchanged in rats exposed to both factors *(1.1 ± 0.1 vs1.2 ± 0.1, P > 0.05)*. However, plasma NO (µM unit) was significantly lower in rats in rats fed a high salt diet alone *(2.4 ± 0.3 vs 0.7 ± 0.1, P<0.001)* and in rats exposed to high environmental temperature *(2.4 ± 0.3 vs 0.3±0.1, P<0.001)* compared to control, but unchanged in rats exposed to a combination of high environmental temperature and high salt diet *(2.4 ± 0.3 vs 1.7 ± 0.2, P > 0.05)*.
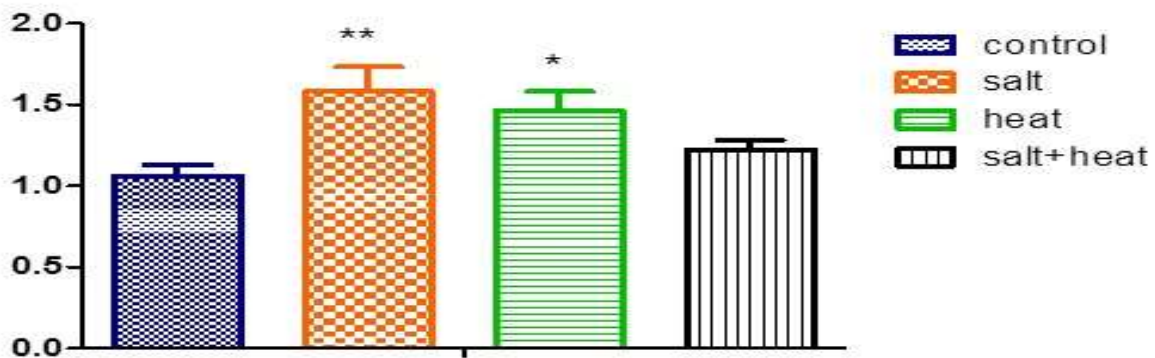
**Figure 2.** Heat shock protein 70 (ng/ml);*P<0.05, **P< 0.01 vs control; Data presented as Mean ± SEM (n=8).
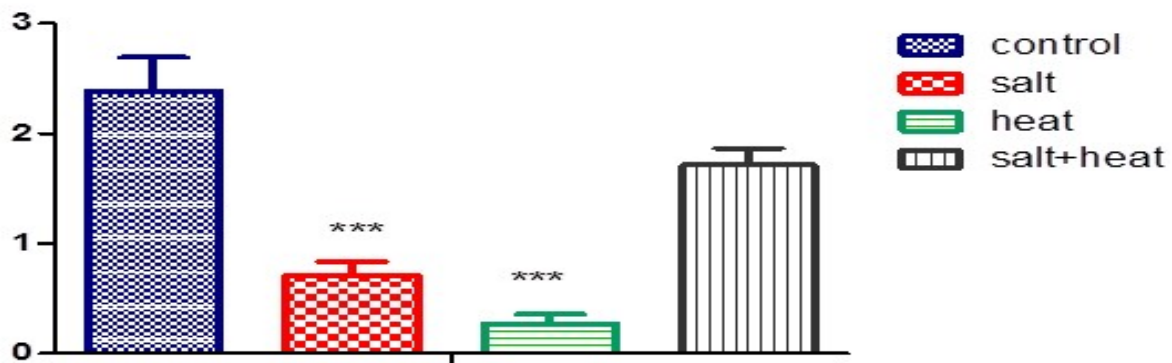
Figure 3. Nitric Oxide (µM unit); ***P < 0.001 vs control. Data presented as mean ± SEM (n=7).

Body weight gain in experimental rats of salt loaded rats exposed to high environmental temperature

High environmental temperature alone and combined with high salt diet significantly ($p<0.05$, $p<0.01$, $p<0.001$) retarded weekly body weight gain in the experimental rats (Figure 4).
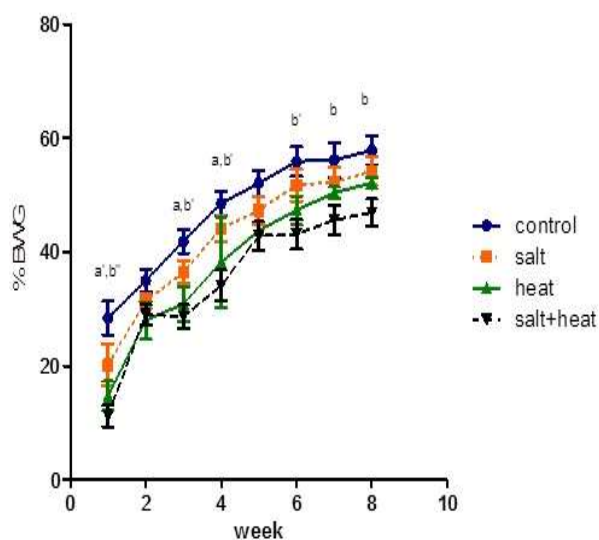
Figure 4. BWG: Body Weight Gain; [a]$p < 0.05$, [a']$p< 0.01$ vs heat; [b]$P < 0.05$, [b']$p < 0.01$, ["b]$p < 0.001$; Data presented as Mean $\pm$ SEM (n=8).

Discussion

High environmental temperature has been described as a silent killer [23], while high salt diet consumption was reported to be responsible for 1.65 million cardiovascular-related global deaths in 2010 [24]. Our previous study showed that high environmental temperature increased the severity of hypertension in male Sprague-Dawley rats fed a high salt diet by increasing salt retention [5]. This current study attempted to elucidate the plausible role played by oxidative stress mechanism and apoptotic markers in driving this outcome. First, we observed that high

environmental temperature combined with high salt diet over a long-term increased the product of oxidative cellular damage, MDA, in blood circulation, with associated increase in p38 MAPK expression in the kidneys of our experimental rats. Next, circulatory NO and HSP70 were unaltered by high environmental temperature in rats fed with high salt diet. Lastly, our results demonstrated that high environmental temperature significantly slowed down weekly body weight gain in rats fed with high salt diet.

Oxidative stress is an important mechanism underlying the pathophysiology of several disease conditions, including cardiovascular disorder [25] and kidney injury [26]. It occurs when there is an imbalance between reactive oxygen species (ROS) and antioxidant defense mechanisms production. The antioxidant defense system, including SOD, CAT, glutathione peroxidase (GPx) and GSH, protect cells against the deleterious effects of ROS namely Superoxide radicals ($O_2\bullet-$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($\bullet OH$) . The depletion of these antioxidants results in progressive oxidative damage to cellular macromolecules, including lipid, proteins and DNA in biological organs. Oxidative damage to membrane lipid in turn increases circulatory MDA which is considered as a marker of lipid peroxidation and cellular damage [27]. In addition, MDA is a highly reactive agent with the ability to attack different cellular macromolecules, including amino acid and sulfhydryl moiety of proteins. This could promote the alterations of biochemical properties of these biomolecules, and subsequently results in the loss of functional protein, modifications of enzymes and carriers proteins, distortion of cytoskeletal units and DNA bases, consequently leading to cell death [28].

In this current study, rats chronically fed with HSD alone or exposed to prolonged HET alone had significantly depleted plasma CAT activity and GSH concentration with raised MDA concentration compared to control rats. Similarly, plasma CAT activity and GSH concentration were depleted in rats fed a high salt diet in combination with exposure to high environmental temperature. However, plasma MDA concentration in rats exposed to the combined environmental factors was significantly higher than the level seen in rats exposed to either of the factors. First, these findings indicate that oxidative stress resulting from prolonged high salt diet consumption alone was comparable to that caused by prolonged exposure to high environmental temperature alone. But most importantly, this present result suggests that interaction between high salt diet and high environmental temperature exacerbates lipid peroxidation and cellular damage as evident by the synergistic rise in circulatory MDA concentration in our experimental rats. In agreement with our findings, earlier studies also demonstrated the contribution of high salt diet [29] and environmental heat [30] to oxidative stress in health and diseases. Meanwhile, previous studies have shown that oxidative stress caused by environmental stressor activates p38 MAPK signaling pathway to mediates DNA damage and cell death [11], while HSP70 is inducted to offer protection against apoptotic cell death [8]. Again, p38 MAPK has been reported as a key driver of HSP70 induction [31]. Most importantly, a protective relationship is reported to exist between the activation of p38 MAPK and the induction of HSP70 [32].

Interestingly, the interaction between HSD and HET in our present study significantly increased the expression of p38MAPK in the renal tubules of our experimental rats, with some observed synergistic effect as seen in the circulatory MDA. The renal expression of p38 MAPK was also increased moderately by exposure to HET but not HSD alone. This is partly in agreement with a study by Hao et al. [33] which showed that thermal stress increases the phosphorylation and the expression of p38 MAPK similar to the action of high-salt diet [34]. This is indeed germane, since p38 MAPK pathway plays key role in the pathophysiology of hypertension [35], renal failure [36] and DNA damage [11]. The molecular actions of MAPK in the kidney [35] and other organs results from its ability to upregulates specific inflammatory cytokines such as IL-6, IL-8, and TNFα in several biological contexts, including kidney damage, myocardial injury [37], cardiomyocytes apoptosis and spontaneous hypertension in stroke prone rats [13]. These actions, in addition with the potential of p38 MAPK to activate SGK1 diet [34], possibly contributed to the increased salt retention caused by the interaction between HSD and HET in our previous study [5]. Specifically, SGK1 increases epithelial sodium channel (ENaC)-mediated Na^+ transport by a number of mechanisms in the kidney, including increased apical membrane localization of ENaC, inhibition of ENaC degradation and stimulation of ENaC transcription [38]. This is indeed critical given the role of ENaC in salt-sensitive hypertension.

Meanwhile, circulatory HSP70 is a reliable indicator of presence of chronic stress, released intracellularly and extracellularly by several environmental stress factors, including thermal and salt-induced stress [7]. Generally, the presence of HSP offers protection against programmed cell death via increase correction of folding of regulatory proteins, reduced activation of p38 kinase [39], inhibition of apoptotic signaling pathways in chronic

diseases [31] and the suppression of caspase-3 activation critical to cell death [40]. But in addition, HSP70 confers thermotolerance on cells previously exposed to hot environment [42], protect the liver against oxidative stress [43], enhances nitric oxide production [9] and increases the chances of animal survival.

In this current study, HSP70 induction was higher in rats exposed to high environmental temperature alone and in rats fed with high salt diet alone compared to control rats. This result is in tandem with earlier investigations which reported similar increase in HSP70 induction by heat stress [44] and high dietary salt [7]. But combined together, HSD and HET did not increase the induction of HSP70 in this present study. This implies a possible antagonistic effect of interaction between HSD and HET on HSP70 induction and the consequent abrogation of the potential protective activity of HSP70 on vital organs. This is supported by studies which showed that the inhibition of HSP70 resulted in the withdrawal of its cytoprotective influence [33], whereas the inhibition of p38 MAPK mitigated the physiological impact of stress [40]. Therefore, the synergistic increase of renal p38 MAPK expression with associated unaltered HSP70 portends double risks in rats exposed to HSD combined with exposure to HET. This thought is supported by the observed retarded body weight gain in this group of rats. Furthermore, in comparison to control rats, circulatory NO was lower in rats fed a HSD alone and in rats exposed to HET alone, with associated rise in HSP70, but was unchanged in rats exposed to both factors with associated unchanged HSP70. The depleted circulatory NO with the converse rise in HSP70 supports a study which suggest that NO inhibition resulted in HSP70 induction [45]. Invariably, the absence of NO inhibition in rats exposed to the combined environmental

factors possibly contributed to the unchanged HSP70 albeit the presence of the dual environmental stressors.

The main limitation of our study includes inadequate resources to evaluate the level of phosphorylation of p38 MAPK expressed in kidneys of the experimental rats. This knowledge could further provide information on the activation of this important cellular marker. In addition, Lack of investigation of inflammatory cytokines upregulated by p38 MAPK was also another limitation in this study.

Conclusion

Overall, our study suggests that prolonged exposure to high environmental temperature may worsen the cardio-renal outcome of long-term consumption of high dietary salt by way of escalating renal expression of p38 MAPK and promoting oxidative cellular damage, with conversely unchanged circulatory HSP70 and NO. These change possibly contributed to the severity of hypertension caused by the two environmental factors as presented in our earlier work. In addition, interaction between high salt diet and high environmental temperature may also exert negative impact on growth. It is not however clear whether or not the increased expression of p38 MAPK was a compensatory response to the unaltered HSP70 induction in rats exposed to the combined environmental stress.

ORCID iD of the author(s)

Francis M. A / 0000-0002-4643-0212
Ahmed K Oloyo / 0000-0003-3442-375X
Arikawe A Paul / 0000-0002-1852-9274
Chikodi N Anigbogu / 0000-0001-9134-3508
Olusoga A Sofola / 0000-0002-5532-0488

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