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THE THERMAL POTENTIATION OF ACETAMINOPHEN-INHIBITED PMN OXIDATIVE METABOLISM *IN VITRO*

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ABSTRACT

The effect of high temperatures (39, 41, and 43 °C) on acetaminophen (AM-) induced inhibition of the oxidative respiratory burst of polymorphonuclear leukocytes (PMNs) *in vitro* has been examined. Whole blood or isolated human PMNs were exposed to various temperatures *in vitro* in the presence or absence of AM for 0–90 min. Phagocyte membrane-bound NADPH oxidase was studied using the luminol chemiluminescence (CL) response and the superoxide dismutase inhibitable reduction of ferricytochrome C. The NADPH oxidase was stimulated by phorbol myristate acetate (PMA). The results showed that high temperatures (39–43 °C) potentiate the AM inhibitory effect on CL peak response of phagocytes in a temperature-dependent manner. Furthermore, the inhibition of superoxide (O_2^-) production induced by AM was potentiated by incubating the cells at 39 or 43 °C at different time intervals. These studies suggest that high temperatures significantly potentiate the AM inhibitory effect on oxidative metabolism of PMNs *in vitro*. These actions of AM may influence the outcome in patients with infectious febrile conditions.

KEY WORDS: human polymorphonuclear leukocytes; heat; chemiluminescence; acetaminophen; superoxide

INTRODUCTION

Polymorphonuclear leukocytes (PMNs) are considered to be the first line of defence against bacterial invasion. Stimulation of membrane-bound NADPH oxidase in PMNs results in the production and release of oxygen free radicals (OFRs), metabolites, superoxide (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radicals (^-OH).[1] In addition, activated PMNs secrete myeloperoxidase (MPO) into the extracellular medium where it catalyses the oxidation of chloride (Cl^-) by hydrogen peroxide (H_2O_2) to yield hypochlorous acid.[2] The oxygen reactive metabolites may be considered to play a major role in the microbicidal action of PMNs.[3]

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The analgesic and antipyretic drug, acetaminophen (AM, N-acetyl-*p*-aminophenol) binds to purified myeloperoxidase (MPO) via its acetamido side chain and paralyses the MPO–H_2O_2–Cl^- antimicrobial system of PMNs.[4] Furthermore, AM is capable of inhibiting superoxide anion generation and the degranulation of PMNs.[5] In a recent study conducted in this laboratory, AM exerted a marked inhibitory effect on the ability of isolated human PMNs to generate OFRs upon stimulation with the soluble agent, phorbol myristate acetate (PMA). Also, AM exerted some inhibitory effect on the ability of PMNs to generate superoxide anions and to phagocytose opsonized dead yeast cells in a dose-dependent fashion.[6]

Modulation of PMN functions by high temperature was reported by several authors. A significant enhancement of bactericidal activity of human PMNs at 40 against 37 °C was noted by Roberts and Steigbigel.[7] In addition, Koller *et al.*[8] reported that incubation of isolated human PMNs at 42 °C inhibited the chemiluminescence (CL) response induced by opsonized zymosan, chemotaxis against C5a, and leukotriene generation by Ca^{2+} ionophore A 23187 or opsonized zymosan but did not affect the viability of PMNs. Similarly, from previous work undertaken in this laboratory, it was found that incubation of PMNs at 38, 39 or 40 °C and subsequent stimulation with PMA or opsonized zymosan caused significant increases in the CL responses. However, pre-incubation of PMNs at temperatures higher than 40 °C produced a temperature-dependent inhibition of the respiratory burst. Besides, at a higher temperature (42 °C) the viability of PMNs was markedly reduced.[6]

Elevation of the body temperature is usually associated with viral or bacterial infections, and, since AM is a widely used antipyretic drug, the present study was undertaken to examine the effect of low concentration of AM (10^{-7} M) on the oxidative metabolism of whole blood as well as isolated human PMNs incubated at temperatures less than 37 °C for various time intervals, using the luminol-dependent CL technique. Additionally, the effect of temperature on AM-induced inhibition of superoxide generation by isolated PMN was investigated using the ferricytochrome C reduction method. In this way, the effect of the combination of heat and AM on PMN viability was examined.

MATERIALS AND METHODS

Reagents

AM (Sigma Chemical Co., St. Louis, MO, U.S.A.) was dissolved in saline 0·9% (w/v). Nycodenz monocyte solution (Nyegaard and Co., AS, Torshov, Norway), is a ready-made sterile solution used for the isolation of PMNs. Luminol was dissolved in dimethyl sulphoxide (DMSO) in a concentration of 10^{-2} M. This stock solution was further diluted in PBS to 10^{-4} M prior to use.

A stock solution of PMA (Sigma Chemical Co.), 2 mg mL^{-1} in DMSO, was prepared and stored in the freezer. This stock solution was further diluted by adding 50 μL PMA stock solution to 10 mL PBS before use. Other reagents used were ferricytochrome C (Sigma Chemical Co.), 160 μM in Earle's balanced salt solution without phenol red (GIBCO, Grand Island, NY, U.S.A.), and superoxide dismutase (Sigma Chemical Co.), 0·2 mg mL^{-1} in PBS.

Isolation of PMNs

Blood was collected by venipuncture from healthy male blood donors in a sterile container containing heparin (10 IU mL^{-1}, Fisher Scientific Co., NJ, U.S.A.). The heparinized blood was mixed with Dextran T500 (6% w/v) and allowed to stand for 30 min at room temperature. The leukocyte-rich plasma layer was removed after erythrocytes had settled. About 5–6 mL of leukocyte-rich plasma was layered over 3 mL Nycodenz solution and centrifuged (Heraues GmbH, Osterode, Germany) at 400 *g* for 10 min at 22 °C. To the bottom PMN-rich portion, 10 mL of PBS was added and centrifuged at 350 *g* for 10 min. Erythrocytes were haemolysed using hypotonic PBS (1:1 with H_2O). The cells were then centrifuged as above, resuspended in PBS to 5×10^6 cells mL^{-1} and examined microscopically for viability.

Measurement of PMN CL response

The principle of oxidation of luminol, 5-amino-2,3-dehydro-1,4-phthalazinedione, by reactive oxygen species (O_2^-, OH^-, and H_2O_2) produced by stimulated PMNs was used to increase the amount of measurable light.[9] The CL assay was carried out in an LKB (WALLAC) 1251 luminometer as described by Kato *et al.*[10] In brief, 100 μL of isolated PMN suspension or whole blood were placed with 400 μL PBS and 100 μL AM (10^{-7} M) in the vial and incubated at the desired temperature (37, 39, 41, or 43 °C) and time interval (0–90 min). Luminol (200 μL) and the stimulant PMA (200 μL) were added after the incubation. The temperature of the luminometer was adjusted according to the incubation temperature. The light output in millivolts was continuously recorded for 20 min and the maximum peak responses were noted.

Measurement of superoxide generation

The reduction of ferricytochrome C by O_2^- is the basic principle of this assay.[11] The amount of reduced ferricytochrome C is determined by measuring its absorbance at 550 nm in the micro-ELISA reader Dynatech MR 580 as described by Pick and Mizel.[12] Briefly, 100 μL PMN suspension in BSS without phenol red, 50 μL AM (10^{-7} M), 100 μL ferricytochrome C (160 μM), and 100 μL PMA (10 μg mL^{-1}) were placed in the microtitre wells. For the control,

AM was replaced by 50 μL saline. 100 μL superoxide dismutase (SOD) (0·2 mg mL^{-1}) and 50 μL saline were used instead of PMA and AM respectively for the blank. The plate was covered and placed in a humidified incubator and gassed with 95% air and 5% CO_2 at the desired temperature (37–43 °C) and time intervals (0–90 min), followed by measurement of the absorbance value for each well. The amount of superoxide anion in nanomoles was calculated as follows:

$$O_2^- \text{ (nmol)} = \frac{(\text{test OD} - \text{assay blank OD}) \times 100}{6{\cdot}3}$$

where OD is the optical density

PMN viability

To exclude a possible cytotoxic reaction due to the combined action of AM (10^{-7} M) and increasing degrees of temperature (37, 39, 41, and 43 °C), the viability of pre-incubated PMNs was tested every 30 min up to 90 min at each temperature. The percentage of viable PMNs was estimated by trypan blue exclusion which was carried out by a microscopic count of cells not stained by 0·2% trypan blue and was expressed as a percentage of unstained cells to total cell numbers.

STATISTICAL ANALYSIS

The data were represented as mean ± standard error (SE) and the differences between the groups were analysed for significance using Student's paired *t*-test. Significance was taken as being $p < 0{\cdot}05$.

RESULTS

The combined effect of heat plus AM (10^{-7} M) on whole blood and on isolated human PMNs: luminol-dependent CL response

Exposure of isolated human PMNs to 39 °C significantly enhanced the CL peak response compared with 37 °C. By contrast, a significant inhibition of CL peak response was observed when PMNs were incubated at higher temperatures (41, 43 °C) (Table 1). Additionally, pre-treatment of PMNs with AM (10^{-7} M) and subsequent exposure to high temperatures (39 or 41 °C) potentiates the inhibitory effect of AM on CL peak responses as shown in Table 1. The percentage of inhibition is temperature dependent. Similar findings were seen when whole blood was incubated with AM and exposed to high temperature (Table 2).

Table 1. The effect of various temperatures with or without AM on the CL of isolated human PMNs stimulated with PMA at different times of incubation. PMN count= 5×10^5 cells mL^{-1}; PMA concentration=2 ng mL^{-1}; luminol concentration=10^{-4} M; AM concentration=10^{-7} M. Each value was expressed as the mean of five experiments ± SE. % CL inhibition=[(without AM − with AM)/without AM]×100

Temperature (°C)	Time of incubation (min)	CL peak response		% inhib.
		Without AM	With AM	
37 (control)	0	222±10·3	167±8·6	25
	30	183±7·9	145±6·9	21
	60	143±12·4	107±4·6	25
	90	123±11·9	100±5·2	19
39	0	281±13·7*	197±9·2*	30
	30	273±14·6**	177±12·8*	35
	60	195±10·2*	140±9·7*	28
	90	162±19·4**	100±9·6	38
41	0	179±20·1**	104±10·5**	42
	30	168±15·3*	84±8·1**	50
	60	100±5·7**	54±6·3**	46
	90	75±5·6**	41±7·4**	45
43	0	150±9·2**	61±4·6**	59
	30	47±5·0**	18±3·1**	62
	60	36±3·2**	15±1·4**	58
	90	23±1·4**	8±0·17**	65

*$p \leqslant 0{\cdot}01$, **$p \leqslant 0{\cdot}001$ compared with control.

The effect of AM (10^{-7} M) combined with heat stress on superoxide production by isolated PMNs

Incubation of isolated PMNs at 39 °C causes a significant increase in superoxide generation, compared with the control (37 °C). On the other hand, higher temperatures, 41 and 43 °C, inhibited superoxide production. The percentage of inhibition was more significant at 43 °C. The inhibitory effect of AM on the production of O_2^- by PMA-stimulated PMNs was significantly intensified by incubating the PMNs at 39, 41, or 43 °C, in a time- and temperature-dependent manner (Table 3).

The effect of various temperatures and AM on the viability of PMNs

Incubation of PMNs at 37, 39 or 41 °C, either alone or in combination with AM (10^{-7} M), did not alter the cell viability. However, incubation of PMNs at 43 °C with or without AM resulted in a remarkable decrease in cell viability to the same degree (Table 4) and in a time-dependent manner.

Table 2. The effect of various temperatures with or without AM on the CL of whole blood human PMNs stimulated with PMA at different times of incubation. PMN count= 5×10^5 cells mL^{-1}; PMA concentration=2 ng mL^{-1}; luminol concentration= 10^{-4} M; AM concentration= 10^{-7} M. Each value was expressed as the mean of five experiments ± SE. % CL inhibition=[(without AM − with AM)/without AM] × 100

Temperature (°C)	Time of incubation (min)	CL peak response		% inhib.
		Without AM	With AM	
37 (control)	0	10·6 ± 1·4	7·5 ± 0·93	29
	30	11·7 ± 2·0	8·4 ± 0·66	28
	60	9·9 ± 1·1	6·9 ± 0·9	30
	90	10·0 ± 0·98	6·7 ± 0·34	33
39	0	12·1 ± 1·9*	7·8 ± 0·7	36
	30	14·9 ± 2·3*	10·4 ± 1·0*	30
	60	14·4 ± 1·4**	8·9 ± 0·97*	38
	90	14·6 ± 2·0*	7·9 ± 0·51*	46
41	0	9·5 ± 1·3	5·7 ± 0·63*	40
	30	10·2 ± 1·4	5·6 ± 0·81**	45
	60	6·0 ± 0·72**	3·5 ± 0·12**	42
	90	4·1 ± 0·2**	2·2 ± 0·13**	46
43	0	6·1 ± 0·43**	3·0 ± 0·4**	51
	30	6·3 ± 1·1**	2·4 ± 0·2**	62
	60	2·3 ± 0·3**	0·8 ± 0·1**	65
	90	1·8 ± 0·1**	0·7 ± 0·03**	61

*$p \leqslant 0{\cdot}01$, **$p \leqslant 0{\cdot}001$ compared with control.

DISCUSSION

The present study shows a strong inhibitory effect of heat on the production of OFRs, induced by the NADPH oxidase stimulus. Incubation at lower temperature (39 °C) significantly enhances phagocytic OFR production but at high temperatures (41 or 43 °C) the effect is significantly inhibited. Stimulation of the NADPH oxidase system is known to be a direct activation of the phospholipid-dependent kinase, protein kinase C.[13,14] This protein kinase in turn activates the membrane NADPH oxidase which is responsible for superoxide generation in PMNs.[15] The stimulated CL response induced by a moderate increase in temperature (39 °C) may be due to the activation of this enzymatic system. It is possible that the stimulation of the oxidative metabolism noted *in vitro* may occur *in vivo*, where it will potentiate the body defences by enhancing the bactericidal activity of PMNs in infectious febrile conditions, in which body temperature is usually around 39 °C.

Maridonneau-Parini *et al.*[16] found that exposure of isolated human PMNs to heat shock above 40 °C stimulated such cells to synthesize heat shock proteins (HSPs) in a temperature-dependent manner. Same authors also observed that HSPs inhibited NADPH oxidase activity. This may be the underlying

Table 3. The influence of different incubation temperatures with or without AM on superoxide production by isolated human PMNs stimulated with PMA. PMN count= 5×10^7 cells mL^{-1}; PMA concentration=2 ng mL^{-1}; ferricytochrome C concentration=160 mM; AM concentration=10^{-7} M. Each cell sample was assayed in triplicate. Each value was expressed as the mean of five experiments ± SE. O_2^- (nmol)=[(test OD − blank OD)/6·3]×100 where OD is the optical density

Temperature (°C)	Time of incubation (min)	Superoxide production nmol/10^6 PMNs		
		Without AM	With AM	% inhib.
37 (control)	0	6·0±0·61	5·5±0·4	8
	30	5·0±0·38	4·5±0·71	10
	60	4·5±0·30	4·0±0·15	11
	90	4·0±0·50	3·6±0·53	10
39	0	6·2±0·42	5·2±0·92	16
	30	6·9±0·25*	4·9±0·31	29
	60	5·0±0·30*	3·9±0·6	22
	90	3·8±0·29	3·0±0·15*	21
41	0	5·6±0·73	4·4±0·9*	21
	30	5·1±0·35	3·7±0·54*	27
	60	3·6±0·17*	2·5±0·1**	31
	90	2·3±0·10**	1·5±0·04**	35
43	0	4·2±0·30*	3·0±0·36**	29
	30	3·5±0·16*	2·3±0·24**	34
	60	2·8±0·27**	1·8±0·41**	36
	90	1·9±0·03**	1·2±0·09**	37

*$p \leqslant 0{\cdot}01$, **$p \leqslant 0{\cdot}001$ compared with control.

mechanism for the inhibited CL response at 41 °C and 43 °C observed in the present study. On the other hand, the marked reduction of the viability of PMNs might explain the strong inhibition of CL response seen at higher temperature (43 °C).

Several studies have shown that the antipyretic drug AM inhibits the functions of PMNs. This takes the form of paralysis of the MPO–H_2O_2–Cl system of PMNs[4] as well as inhibition of superoxide production. Degranulation of isolated human PMNs was also reported after treatment with AM.[5] In this laboratory, AM was found to inhibit the CL response of PMNs, superoxide production, and phagocytosis of opsonized dead yeast cells.[6] The present study extends these observations further. Thus the addition of AM not only counteracted the stimulating effect of 39 °C on isolated PMNs, but also increased the percentage of inhibition of OFR production compared with the control (37 °C). Besides, the percentage of OFR inhibition of AM-treated cells at the higher temperatures, 41 or 43 °C, increased further, suggesting that AM inhibits the respiratory burst of PMNs in a temperature-dependent manner. These observations suggest that high temperatures potentiate the mechanism(s) responsible for the AM inhibitory effects on

Table 4. The efect of different incubation temperatures with or without AM on the viability of human PMNs. PMN count= 5×10^6 cells mL^{-1}; AM concentrations= 10^{-7} M. Viability was examined by trypan blue exclusion. The numbers between brackets represent the percentage viability of PMNs without AM treatment

Incubation time (min)	Percentage viability incubation temperatures			
	37 °C	39 °C	41 °C	43 °C
0	97 (98)	97 (96)	96 (95)	95 (96)
30	95 (97)	96 (94)	94 (96)	80 (81)
60	91 (95)	92 (95)	91 (93)	74 (73)
90	93 (92)	91 (93)	89 (90)	68 (63)

PMNs. A similar inhibitory effect was obtained when whole blood was used instead of isolated PMNs and this indicates that no other blood components interfere with these mechanisms. The results presented in this study do not explain the mechanism of the potentiation of the inhibitory activity of AM.

The results of this study may also shed some light on the therapeutic action of AM in heat febrile conditions. The concentration of AM (10^{-7} M) used in this study is lower than the therapeutic plasma level of AM, 10–20 $\mu g\, mL^{-1}$, which is equivalent to 7×10^{-5}–10^{-4} M.[17] Thus, when AM is given to patients with hyperthermia associated with infections, it may produce significant inhibition of the bactericidal activity of PMNs. This may be of special significance if such patients are already immuno-compromised.

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ABBREVIATIONS

PMN	polymorphonuclear leukocytes
O_2^-	superoxide
H_2O_2	hydrogen peroxide
MPO	myeloperoxidase
Cl^-	chloride
AM	acetaminophen
OFRs	oxygen free radicals
PMA	phorbol myristate acetate

CL chemiluminescence
PBS phosphate buffered saline
SOD superoxide dismutase
HSPs heat shock proteins
DMSO dimethyl sulphoxide

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