Potentiation of the Thermogenic Antiobesity Effects of Ephedrine by Dietary Methylxanthines: Adenosine Antagonism or Phosphodiesterase Inhibition?

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Current concepts about the mechanisms underlying the therapeutic effects of dietary methylxanthines (caffeine, theophylline, and theobromine) favor their actions as antagonists of adenosine receptors, and attribute their other possible modes of action, namely those associated with translocation of intracellular calcium, inhibition of phosphodiesterase enzyme (PDE) activity, or the release of catecholamines, to high (near-toxic) doses. From studies measuring the respiration rate of brown adipose tissue (BAT), evidence is provided here that at concentrations compatible with therapeutic doses, the ability of methylxanthines (25 to 50 μmol/L) to potentiate the thermogenic effect of the sympathomimetic drug, ephedrine (0.25 μmol/L), particularly under conditions of caloric restriction, involves a minor contribution of adenosine antagonism, but could mainly be explained by the inhibition of PDE activity. In view of current interest in the pharmacological stimulation of metabolic rate to assist the management of obesity with low-calorie regimens, the targeting of PDE activity is therefore a rational approach in the search for drugs that could potentiate sympathomimetic stimulation of metabolic rate.

THE PHARMACOLOGICAL approach to enhancing metabolic rate in the management of obesity has been the subject of considerable interest over the past decade.[1-2] The search for potential thermogenic drugs has centered on the development of novel β_3-adrenoceptor agonists,[3] but attention has also focused on the methylxanthines because they are considered to be relatively "safe" drugs capable of interfering with the adrenergic system for thermogenic stimulation.[4-6] Indeed, they are widely consumed in the form of many beverages (coffee, tea, cocoa, cola), chocolates, and cakes and, in addition, they are commonly found in preparations (often over-the-counter) for coughs, asthma, bronchospasm, analgesia, apnea, etc. Although in acceptable doses, the thermogenic effects of these methylxanthines seem to be too mild for obesity therapy,[7] current interest focuses on their ability to potentiate the thermogenic effect of ephedrine—a sympathomimetic with both anorectic and thermogenic properties—which has been shown to enhance weight loss in diet-restricted patients.[8-11] The interaction between ephedrine and caffeine on whole-body thermogenesis has now been confirmed in man,[12,13] and combinations of these drugs have been shown to be safe and more effective than either ephedrine or caffeine alone in facilitating weight loss.[14]

In an attempt to gain insight into the peripheral mechanisms of thermogenesis induced by these drugs, we have conducted a series of studies that assess in vitro the rate of O_2 consumption of the rat interscapular brown adipose tissue (IBAT), a tissue richly innervated by sympathetic nerves and whose respiration rate is a sensitive index of thermogenesis.[15] Using intact (innervated) and denervated tissues, we recently reported that both ephedrine (<1 μmol/L) and caffeine (<2 mmol/L) activated thermogenesis indirectly by enhancing the release of noradrenaline (NA) from sympathetic nerves, and that direct postsynaptic actions were only present at higher drug concentrations.[16] In addition, these studies also demonstrated a permissive effect of caffeine in allowing a subthreshold dose of ephedrine to activate thermogenesis. This interaction could be explained by ephedrine's enhancement of sympathetic neuronal release of NA together with caffeine's dual ability to antagonize adenosine-inhibitory effects on NA release and actions and to inhibit phosphodiesterase enzyme (PDE) activity. The net result would be an elevated cellular level of cyclic adenosine monophosphate, a critical intracellular mediator (second messenger) for the actions of catecholamines on thermogenesis.

However, the important question as to which of these two mechanisms contributes mainly to this permissive or potentiative effect of caffeine on ephedrine-induced thermogenesis, specifically in the dose range normally found in plasma, remains to be answered. Current theories attempting to explain the diverse pharmacological actions of methylxanthines tend to favor their main mode of action as antagonists of adenosine actions rather than as inhibitors of PDE activity.[17] This is based on evidence that the maximal therapeutic plasma concentration of theophylline (50 μmol/L) or caffeine (100 μmol/L) is well below their threshold concentration for an inhibitory effect on PDE activity, but, in contrast, is above that for antagonizing adenosine actions.[18] For example, at the therapeutic level for treating asthma, theophylline has been shown to inhibit less than 10% of PDE activity, but 100% of the adenosine response.[19] These findings are thus compatible with the notion that adenosine, an end product of adenosine triphosphate hydrolysis that is released by most cells following stimulation, functions either as a local hormone, messenger, or retaliatory metabolite in the modulation of numerous physiological processes including vascular tone, hormone action, neural function, platelet aggregation, and lymphocyte differentiation.[20] Specifically relevant to adipose tissue metabolism, adenosine has been shown to be a potent antilipolytic and antithermogenic agent in both white and brown adipose tissue (BAT)—effects believed to be mediated either by reducing adipose tissue sensitivity to

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NA and/or by inhibiting NA release from sympathetic terminals.[21-23] However, the factor(s) regulating release of adenosine under physiological conditions remains to be established. Nonetheless, a sympathetic component in the control of adenosine release has been postulated on the basis that following sympathetic nerve stimulation or NA administration, the rate of adenosine release is increased, resulting in a significant inhibition of lipolysis.[24-25] Thus antagonism of adenosine actions has been postulated as the main underlying mechanism by which dietary methylxanthines, in therapeutic doses, potentiate ephedrine-induced thermogenesis.[26]

To test this hypothesis, we have extended our previous investigations using the BAT model, and measured its respiration rate in response to combinations of ephedrine and xanthines specifically at concentrations compatible with therapeutic plasma levels. At the same time the present studies, conducted in tissues from both fed and fasted animals, also examined the relative potencies of the three dietary methylxanthines, caffeine, theophylline, and theobromine, in their interaction with ephedrine in enhancing thermogenesis.

MATERIALS AND METHODS

Animals and Diets

All studies were conducted on male Sprague–Dawley rats (CMU, Geneva, Switzerland) aged 7 to 8 weeks and housed in a temperature-controlled room (23°C) with a 12-hour, light-dark cycle. The animals had free access to water and a standard laboratory chow diet (Provimi-Lacta, Cossonay, Switzerland) consisting (wt/wt) of 20% protein, 60% carbohydrate, and 4% fat. While fasting, animals were given a hypotonic saline solution (0.45%) to drink.

Chemical Denervation

Chemical sympathectomy was performed using 6-hydroxydopamine ([6-OHDA] Sigma, St Louis, MO) dissolved in distilled water containing 0.001 *N*-HCL and equilibrated with nitrogen. Rats were injected subcutaneously with 6-OHDA (50 mg/kg body weight [BW]) twice a day (8:00 AM and 5:00 PM), and were killed 15 hours after the second injection. Such pretreatment with 6-OHDA is well known to markedly deplete NA stores in a variety of sympathetically innervated tissues, and previous studies in our laboratory have reported that our method of inducing chemical sympathectomy results in reductions in catecholamine content of IBAT to below the lowest detectable level.[27] In addition, as described previously,[16] after each in-vitro measurement of tissue respiration rate, a normal or greater maximal response to exogenous NA administration in our IBAT preparation is used to verify that any lack of response to sympathomimetic drugs cannot be attributed to postsynaptic tissue damage following 6-OHDA treatment, but is due to the depletion of NA stores following chemical sympathectomy.

Tissue Preparation

Animals were killed by decapitation between 7:30 and 8:00 AM, and two fragments 10 to 12 mm long, approximately 1 mm thick, and 10 to 14 mg wet weight of IBAT were rapidly dissected out from the middle part of one fat pad. The tissues were perifused with Krebs-Ringer bicarbonate buffer of the following composition (mmol/L): NaCl 116.8, $NaHCO_3$ 25, KCl 5.9, $MgSO_4$ 1.2, NaH_2PO_4 1.2, $CaCl_2$ 1.25, with streptomycin 50 mg/L and glucose (5 mmol/L). The medium was gassed continuously with a mixture of 95% O_2 and 5% CO_2, and was maintained at a set temperature of 30° ± 0.2°C. The choice of 30°C is based on earlier studies[27] indicating that stable respiration and stability of our tissue preparation lasted much longer at 30°C (namely, for 8 to 10 hours) than at 37°C (< 4 hours).

Measurement of Tissue Respiratory Rate

The respiratory rates of IBAT fragments were measured as described by Barde et al.[27] This involves repeated O_2 uptake determinations, based on the principle of Vieira et al.[28] The P_{O_2} of a bubble-free liquid phase enclosed in a thick-walled Lucite chamber was measured by a Clark O_2 electrode connected to a polarographic circuit whose output voltage is directly proportional to P_{O_2}. The formula for calculating the O_2 uptake rate (M_{O_2}) in nmol/mg wet tissue/h has been described previously.[28] All M_{O_2} values were taken during steady-state respiration; this was reached after 90 to 120 minutes for basal respiration, and after 40 to 90 minutes when drug administration resulted in changes in M_{O_2}. In cases where addition of drugs did not alter respiration rate, M_{O_2} values were taken after at least 90 minutes. From our data on tissue respiration rate, it is also possible to estimate the rate of oxygen consumption expressed on a per cell basis. Given that the mean size of a brown adipocyte is 20 μm, and that adipocytes constitute some 40% of the total cell number in this tissue, it can be calculated that there are some 40×10^6 cells/g tissue. The basal respiration rate reported in this study is approximately 40 nmol O_2/mg tissue/h, ie, approximately 17 nmol $O_2/10^6$ cells/min. After correction for temperature differences (Q_{10} = 2 to 2.5) between studies, the value at 37°C would be approximately 25 to 30 nmol $O_2/10^6$ cells/min. This is reasonably close to measured values on brown adipocytes reported in the literature,[29] namely, 34 nmol $O_2/10^6$ cell/min.

Pharmacological Compounds Added to Perifusion Medium

L-Ephedrine hydrochloride (Sigma) was either dissolved in medium or added to medium by means of motor-driven syringes connected to needles set at the inlet of each chamber. Caffeine, theobromine, theophylline, isobutyl-methylxanthine (IBMX), 8-phenyltheophylline (8-PT), 3-propylxanthine ([3-PX] all from Fluka, Buchs, Switzerland), and adenosine deaminase (Boehringer, Mannheim, Germany) were added to the medium by means of motor-driven syringes.

Data Analysis

All data are presented as means ± SEM. Statistical analysis was performed using ANOVA, and pairwise comparisons between treatments were made using Duncan's multiple-comparison test.

RESULTS

Dose-Response Curve for Ephedrine in IBAT From Fed, 1-Day Fasted, and Sympathectomized Animals

We had previously examined the dose-response of ephedrine in IBAT M_{O_2} at 0.001, 0.01, 0.1, 1, 10, and 100 μmol/L, and had shown that ephedrine had little or no effect on M_{O_2} at or below 0.1 μmol/L, but stimulated respiration maximally from 1 μmol/L.[16] The dose-response study is repeated here, but with additional concentrations between 0.1 and 1 μmol/L to examine the profile of the curve in this zone that corresponds to plasma levels of the sympathomimetic when administered therapeutically.

As shown in Fig 1, ephedrine stimulated IBAT M_{O_2} in an

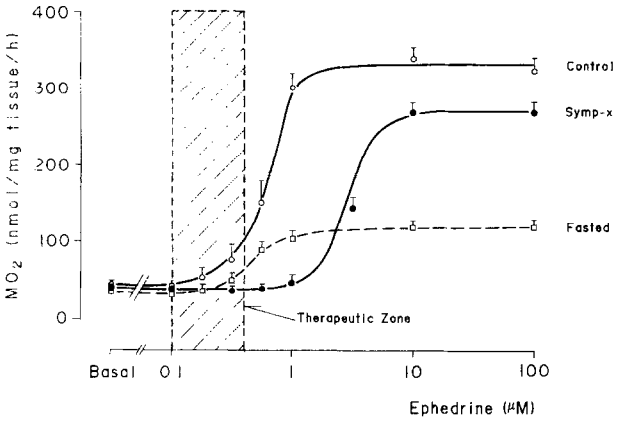

Fig 1. Dose-response of ephedrine on Mo_2 of IBAT fragments from control, chemically sympathectomized (symp-X), and 24-hour–fasted rats. All values are means with vertical bars representing SEM (N = 5 to 8).

exponential manner between 0.1 and 1 μmol/L, and maintained maximal tissue respiration rates (six times basal levels) between 1 and 100 μmol/L. In tissues from animals pretreated with 6-OHDA, a procedure that destroys sympathetic nerve endings and depletes NA stores, the dose-response curve was shifted to the right by a factor of 10 and, in addition, showed a reduction in the maximal tissue MO_2 (control 330 *v* symp-X 270 nmol/mg tissue/h; $P < .01$). Thus, within the therapeutic dose range (0.2 to 0.6 μmol/L), the stimulation of IBAT thermogenesis by ephedrine is entirely dependent on the presence of intact nerve terminals, and is hence mediated indirectly by release of NA from sympathetic nerve terminals.

The dose-response curve of ephedrine was also studied in tissues from animals fasted for 24 hours, a condition well known to reduce thermogenic responsiveness to catecholamines. As shown in Fig 1, the 24-hour fast markedly reduced maximal tissue response to the sympathomimetic by nearly threefold (control 330 *v* fasted 120 nmol/mg tissue/h; $P < .001$). In contrast, basal MO_2 was only slightly and nonsignificantly reduced.

Permissive Influence of Low Doses of Methylxanthines on Effect of Ephedrine in Stimulating IBAT Respiration

This study compared the ability of methylxanthines at low concentrations ranging from 10 to 100 μmol/L to stimulate IBAT MO_2 in response to a subthreshold (and hence ineffective) dose of ephedrine (0.25 μmol/L). As shown in Fig 2, neither methylxanthines (10 to 100 μmol/L) nor ephedrine (0.25 μmol/L) alone were capable of stimulating IBAT MO_2. However, when added in combination with the subthreshold dose of ephedrine, methylxanthines increased IBAT MO_2 in a dose-dependent fashion up to fourfold basal values. The relative potencies of methylxanthines at both 25 and 50 μmol/L were as follows: theophylline > caffeine > theobromine; at 100 μmol/L, theophylline > caffeine = theobromine.

Comparison Between Methylxanthines and Other More Specific Inhibitors of Adenosine Actions or PDE Activity in Their Interaction With Ephedrine in Stimulating MO_2 of IBAT From Fed Animals

The relative importance of adenosine antagonism and PDE inhibition in the interaction between methylxanthines and ephedrine on IBAT MO_2 was subsequently examined. Experiments were performed to assess the extent to which specific inhibitors of PDE or adenosine actions interact with ephedrine (0.25 μmol/L) in the stimulation of IBAT MO_2. Like dietary methylxanthines, the specific inhibitors were without effect on basal MO_2 (Fig 3), but they showed differences in the magnitude of tissue response when added in combination with ephedrine. At equimolar concentrations (25 or 50 μmol/L), 3-PX, a specific PDE inhibitor (with low potency as an adenosine-receptor antagonist), interacted with ephedrine to produce a 2.5- to 3.5-fold increase in MO_2, whereas the addition of 8-PT, a xanthine

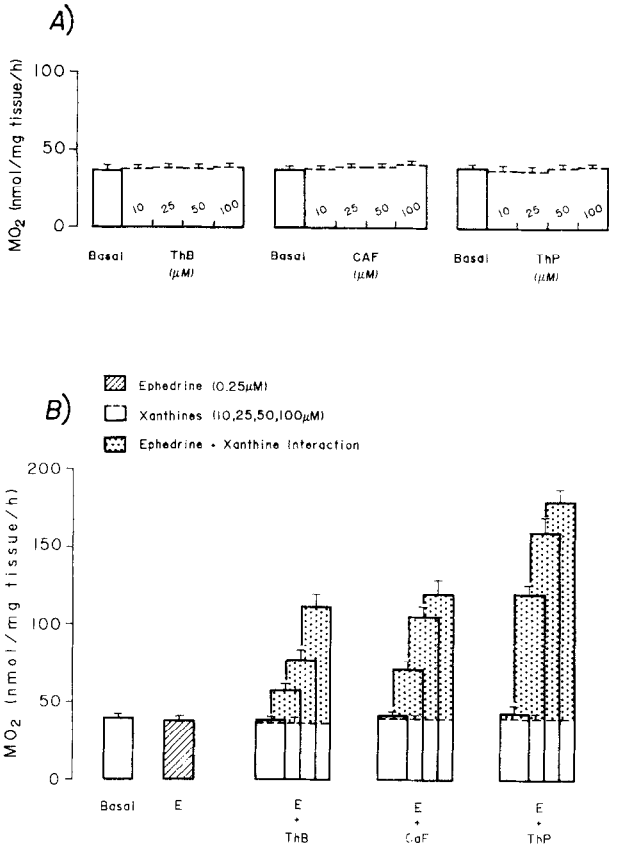

Fig 2. (A) Influence of low concentrations of dietary methylxanthines (10 to 100 μmol/L) on respiratory rate of IBAT from fed animals. ThB, theobromine; CaF, caffeine; ThP, theophylline. Values represent means ± SEM (n = 8 to 12). At these low concentrations, methylxanthines alone had no effect on basal respiratory rate. (B) Interaction between subthreshold concentration of ephedrine (E, 0.25 μmol/L) and different dietary methylxanthines (10 to 100 μmol/L, from left to right) on respiratory rate of IBAT from fed animals. ThB, theobromine; CaF, caffeine; ThP, theophylline. Values represent means ± SEM (n = 8 to 12). ANOVA indicates significant main effects of xanthine type ($F = 25.2$, $P < .001$) and xanthine concentration ($F = 46.7$, $P < .001$), and a significant type × concentration interaction ($F = 2.45$, $P < .05$).

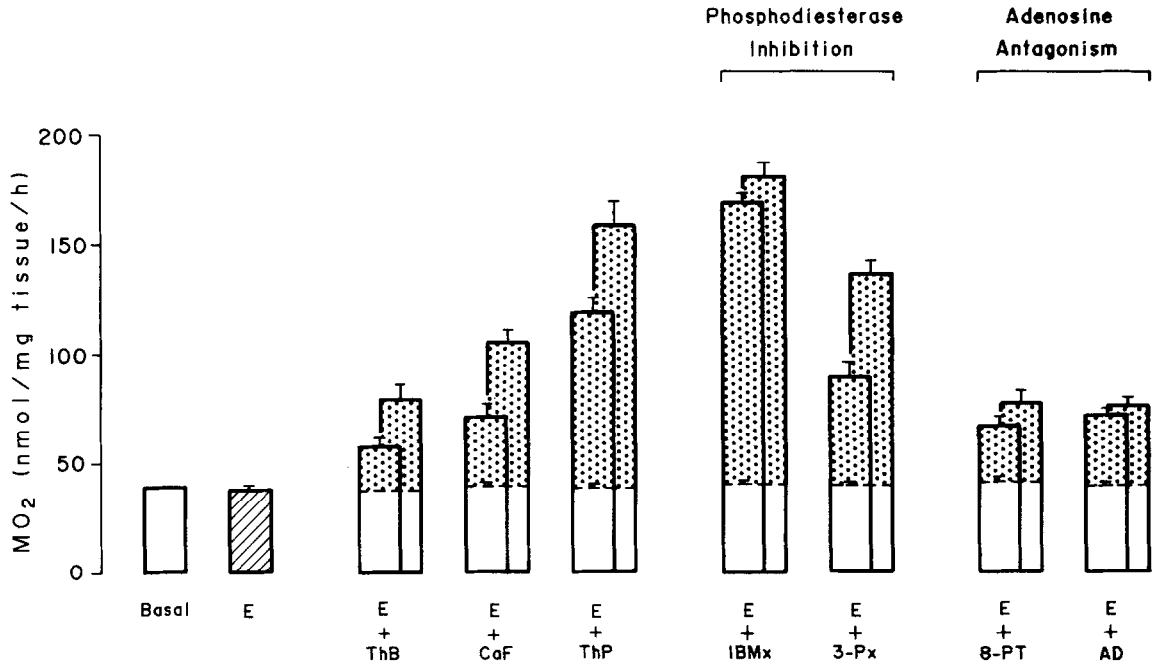

Fig 3. Respiratory rate of IBAT from fed animals in response to subthreshold concentration of ephedrine (E, 0.25 μmol/L) in combination with (1) xanthine analogues with high specificity (3-PX) and high potency (IBMX) for inhibition of PDE enzyme activity, and (2) a xanthine analogue with high specificity for antagonizing adenosine receptors (8-PT), or with adenosine deaminase (1 and 10 μg/mL, from left to right). These data have been compared statistically with data for ephedrine in combination with different dietary methylxanthines at equimolar concentrations (25 and 50 μmol/L, from left to right) to xanthine analogues. ▨, Ephedrine, 0.25 μmol/L; □, xanthines, 25 and 50 μmol/L; ▯, ephedrine + xanthine interaction. ThB, theobromine; CaF, caffeine; ThP, theophylline; AD, adenosine deaminase. Values represent means ± SEM (n = 8 to 12). ANOVA indicates significant main effects of xanthine type ($F = 29.4$, $P < .001$) and xanthine concentration ($F = 18.6$, $P < .001$) and a significant type × concentration interaction ($F = 3.39$, $P < .01$). For both xanthine concentrations (25 or 50 μmol/L), Duncan pairwise comparisons (tested at .05 as the level for statistical difference) indicate the following order of potency: IBMX > ThP > 3-PX > CaF > ThB, 8-PT, AD.

analogue with high potency as an adenosine-receptor antagonist, interacted with ephedrine to cause a less than twofold increase in MO_2. Similarly, addition of adenosine deaminase (for enzymatic inhibition of adenosine production) to ephedrine resulted in a less than twofold stimulation of IBAT MO_2. In contrast, IBMX, a potent inhibitor of PDE activity, in equimolar concentrations to the other xanthines (ie, 25 or 50 μmol/L) was ineffective in alone, but interacted with ephedrine to increase basal MO_2 by more than fourfold. Thus, in the interaction between xanthines and ephedrine, the inhibition of adenosine action (by adenosine deaminase or 8-PT) could account for most or all of the effects with theobromine (25 or 50 μmol/L) and caffeine (25 μmol/L), but, in contrast, it accounts for less than 50% of the effects with caffeine (50 μmol/L) and theophylline (25 or 50 μmol/L), and for less than 25% of the thermogenic effects with IBMX (25 or 50 μmol/L).

Comparison Between Methylxanthines and Other More Specific Inhibitors of Adenosine Action or PDE Activity in Their Interaction With Ephedrine in Stimulating MO_2 of IBAT From Animals Fasted for 24 Hours

The above study using tissues from fed animals was repeated with tissues from animals fasted for 24 hours (Fig 4). The basal MO_2 was only slightly reduced by 1 day of fasting, and neither ephedrine at 0.25 μmol/L nor dietary methylxanthines at 25 and 50 μmol/L had any effect on basal MO_2 by themselves. On the other hand, although of lower magnitude than in the fed state, interactions between methylxanthines and ephedrine persisted in tissues from 1-day–fasted animals, and the relative potency of dietary methylxanthines (25 and 50 μmol/L) was qualitatively similar to that shown for tissues from fed animals, ie, theophylline > caffeine > theobromine. In the presence of ephedrine, theophylline increased basal MO_2 by 2.2- and 3.5-fold, caffeine by 30% and 95%, and theobromine by 0% and 60% at xanthine concentrations of 25 and 50 μmol/L, respectively.

In tissues from fasted animals, various inhibitors with specificity for adenosine antagonism or PDE inhibition showed marked differences in the magnitude of tissue MO_2 response when added to ephedrine. As shown in Fig 4, addition of the specific PDE inhibitor, 3-PX, to ephedrine increased basal MO_2 by twofold to 2.6-fold, whereas the specific adenosine antagonist, 8-PT, or adenosine deaminase, when added to ephedrine, failed to increase MO_2 significantly. This contrasts with the addition of the potent PDE inhibitor, IBMX, to ephedrine, which resulted in a 2.5- to 3.5-fold increase in IBAT MO_2. Thus, in the fasted state, inhibition of adenosine or antagonism of its actions (by adenosine deaminase and 8-PT, respectively) cannot account for increases in MO_2 resulting from the interaction between ephedrine and methylxanthines.

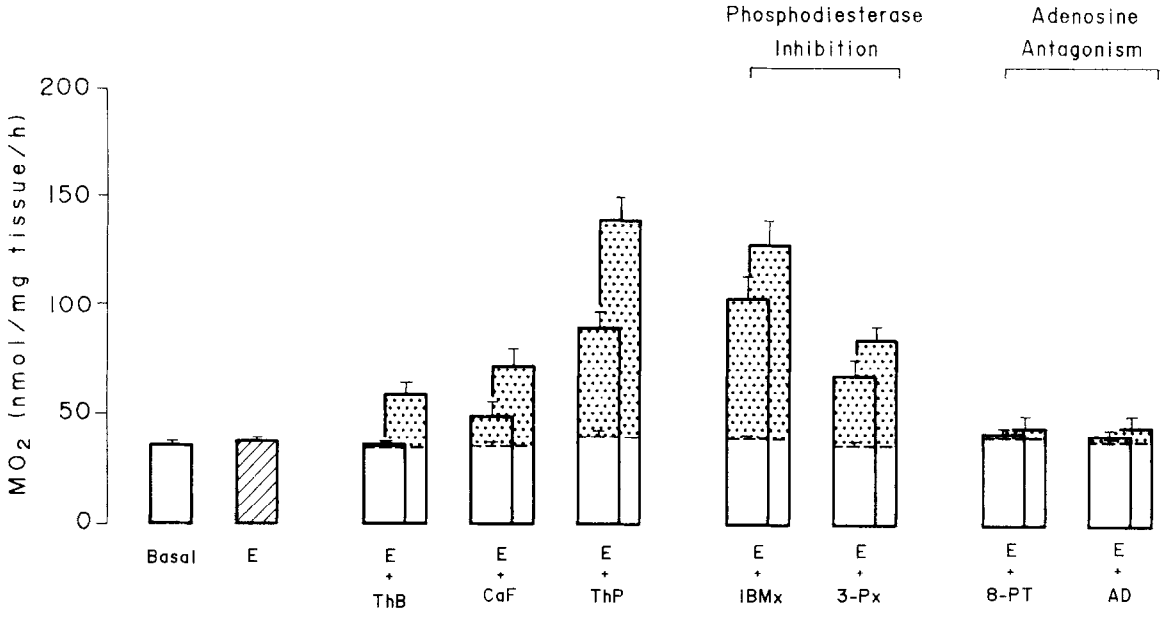

Fig 4. Respiratory rate of IBAT from 24-hour–fasted animals in response to subthreshold concentration of ephedrine (E, 0.25 μmol/L) in combination with (1) different dietary methylxanthines (25 and 50 μmol/L, from left to right), (2) equimolar concentrations of xanthine analogues with high specificity (3-PX) and high potency (IBMX) for inhibition of PDE enzyme activity, and (3) a xanthine analogue with high specificity for antagonizing adenosine receptors (8-PT), or with adenosine deaminase (1 and 10 μg/mL, from left to right). ▨, Ephedrine, 0.25 μmol/L; □, xanthines, 25 and 50 μmol/L; ⊡, ephedrine + xanthine interaction. ThB, theobromine; CaF, caffeine; ThP, theophylline; AD, adenosine deaminase. Values represent means ± SEM (n = 8 to 12). ANOVA indicates significant main effects of xanthine type ($F = 57.0$, $P < .001$) and xanthine concentration ($F = 39.2$, $P < .001$) and a significant type × concentration interaction ($F = 4.02$, $P < .01$). Duncan pairwise comparisons (tested at .05 as the level for statistical difference) indicate the following order of potency: xanthine concentration 25 μmol/L, IBMX, ThP > 3-PX > CaF > ThB, 8-PT, AD; xanthine concentration 50 μmol/L, IBMX, ThP > 3-PX, CaF > ThB > 8-PT, AD. Mo_2 values in response to E + 8-PT and E + AD were not significantly different from basal values.

Interaction Between Ephedrine and Inhibitors of Both PDE Activity and Adenosine Action on MO_2 of IBAT From Fed and Fasted Animals

This additional study investigated the effect of ephedrine on MO_2 during concomitant inhibition of both PDE (using 3-PX) and adenosine (using adenosine deaminase). The results, shown in Fig 5, indicate that the combination of ephedrine and 3-PX increased MO_2 by more than threefold in tissues from fed animals, and by twofold in tissues from 1-day–fasted animals. However, under both fed and fasted conditions, the synergistic interaction between ephedrine and 3-PX was not further augmented by addition of adenosine deaminase.

DISCUSSION

Following therapeutic administration of ephedrine/methylxanthine preparations shown to enhance whole-body thermogenesis in man,[12,13] the plasma level of ephedrine is likely to vary in the range of 0.2 to 0.6 μmol/L,[30] and methylxanthines vary in the range of 10 to 50 μmol/L.[17,19] With emphasis on such concentrations compatible with therapeutic doses, the current studies on BAT fragments confirm our previous findings[16] that ephedrine exerts its peripheral thermogenic effect entirely via the release of NA from sympathetic nerve terminals. These findings are compatible with reports that ephedrine at low concentrations has little or no direct activity in various tissues,[31,32] and that depletion of NA stores with reserpine largely abolished in-vivo thermogenic response to ephedrine.[33] In addition, the current studies demonstrate for the first time the following: (1) methylxanthines in concentrations as low as 25 to 50 μmol/L (and ineffective alone) retain the ability to enable a subthreshold dose of ephedrine to stimulate tissue thermogenesis; (2) dietary methylxanthines differ markedly in the magnitude of their synergistic interaction with ephedrine for thermogenic stimulation, with the order of potency being theophylline > caffeine > theobromine; and (3) interaction of these methylxanthines with ephedrine is mediated primarily by inhibition of PDE activity rather than by antagonizing adenosine actions. The present findings refer specifically to the interaction between these drugs and peripheral sympathetic nerves in BAT. Under in-vivo conditions, ephedrine and methylxanthines may also exert their synergistic effects via central and other peripheral mechanisms (eg, by enhancing adrenal medullary secretion of epinephrine), and the total contribution of other organs and tissues to such sympathomimetic stimulation of thermogenesis is probably more important quantitatively than BAT. Indeed, results of a single-dose study in man suggest that skeletal muscle rather than BAT is the major site of ephedrine-induced thermogenesis.[34] However, given other evidence[35] that atrophied BAT in the adult man can be reactivated (and can indeed proliferate) in response to chronic catecholamine stimulation—eg, in response to cold exposure, or in the presence of the NA-releasing tumor, pheochromocytoma—the possibility remains that following

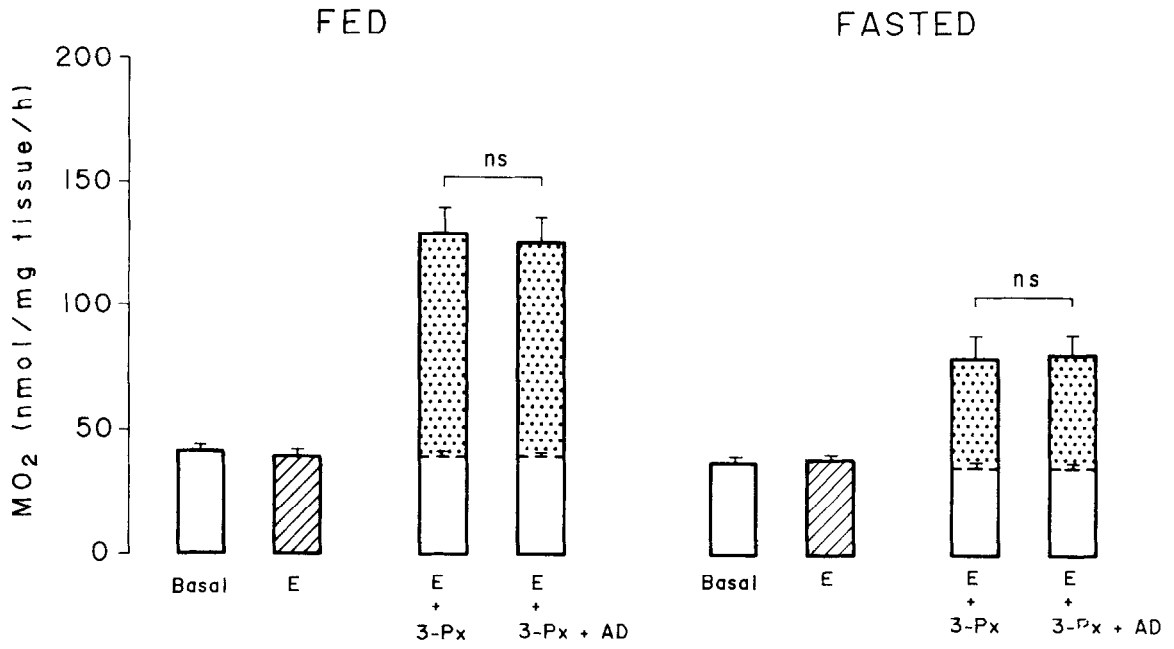

Fig 5. Respiratory rate of IBAT from fed and 24-hour–fasted animals in response to subthreshold concentration of ephedrine (E, 0.25 μmol/L) in combination with 3-PX (50 μmol/L) or with both 3-PX (50 μmol/L) and adenosine deaminase (AD, 10 μg/mL). ▨, Ephedrine, 0.25 μmol/L; □, 3-PX, 50 μmol/L, and/or adenosine deaminase, 10 μg/mL; ⊡, interactions.

long-term administration of such catecholaminergic drugs, BAT may assume a greater importance than that reported in the short-term study. Whether inhibition of PDE, as shown here in rat tissue, is also an important mechanism underlying the potentiative effects of methylxanthines on ephedrine-induced thermogenesis in humans remains to be investigated; but kinetic studies, at least for adipose tissue, have indicated that Michaelis-Menten constants (K_m) for PDE activity in rats and humans are quite similar, namely, a high K_m of approximately 0.04 mmol/L and a low K_m of 0.4 to 0.8 μmol/L occurs in both species.[36] Taken together, results obtained here about the peripheral mechanisms of interaction between ephedrine and methylxanthines in BAT could conceivably be extended to organs and tissues capable of responding to sympathomimetic stimuli.

Several lines of evidence presented here favor PDE inhibition rather than adenosine antagonism as the main mode of action by which methylxanthines exert their permissive influence on ephedrine for BAT thermogenic activation. First, in combination with ephedrine, 8-PT, a xanthine analogue with high specificity for antagonizing adenosine receptors,[37] produced a thermogenic response that is smaller in magnitude than those of equimolar concentrations of either caffeine, theophylline, or the xanthine analogue, 3-PX, a more specific inhibitor of PDE activity. Second, this weaker effect of adenosine antagonism cannot be attributed to the low solubility of 8-PT (and hence to inadequate concentrations of this xanthine reaching the receptors), since enzymatic inactivation of adenosine with adenosine deaminase also led to increases in MO_2 that were smaller in magnitude than those found with either caffeine, theophylline, or the more specific PDE inhibitor, 3-PX.[38] It should also be noted that slow penetration of the tissue by 8-PT or adenosine deaminase cannot be a factor explaining their mild interaction with ephedrine, because their stimulatory effects reached a plateau within an hour, showed no sign of further increasing with time, and remained stable for several more hours. Third, in the fasted state, addition of either 8-PT or adenosine deaminase to ephedrine resulted in little or no stimulation of IBAT MO_2, compared with the several-fold increase in MO_2 obtained when ephedrine was combined with methylxanthines or 3-PX. Fourth, our studies also show a good positive correlation between the potency of caffeine, theophylline, and IBMX as inhibitors of PDE activity and their magnitude of thermogenic stimulation when combined with ephedrine, namely, IBMX > theophylline > caffeine. As for theobromine, its relative potency as an inhibitor of PDE is less well known, but it is generally believed to be less potent than theophylline[19] and hence compatible with this relationship. Finally, it may be argued that the magnitude of thermogenic stimulation of methylxanthines in combination with ephedrine also correlates well with their potencies as adenosine antagonists. However, the fact that under both fed and fasted conditions synergistic interaction between ephedrine and the specific PDE inhibitor, 3-PX, was not further augmented by addition of adenosine deaminase suggests that the potencies of these methylxanthines in their interaction with ephedrine reflect their potencies as inhibitors of PDE activity, per se, rather than those due to an interaction between PDE inhibition and adenosine antagonism. Taken together, our current studies therefore strongly suggest that the permissive or potentiative effect of low concentrations of dietary methylxanthines on the ef-

fects of ephedrine for thermogenic stimulation is explained by ephedrine's enhancement of NA release from sympathetic nerves, together with the ability of methylxanthines to counter negative feedback inhibition of PDE on cyclic adenosine monophosphate production.

The minor importance of adenosine antagonism in this interaction between methylxanthines and ephedrine is compatible with results of a recent report indicating that unlike caffeine, theophylline, and IBMX, adenosine deaminase was weakly permissive for β-adrenergic inhibition of lipogenesis in porcine adipose tissue slice,[39] but is contrary to previous reports in favor of a predominant role for adenosine antagonism in the therapeutic effects of methylxanthines.[17-20] However, it should be noted that much of the evidence favoring adenosine antagonism as the main mode of action of xanthines and, by extension, attributing an important physiological role for adenosine in regulation of adipose tissue metabolism derives from studies on isolated adipocytes as opposed to our studies using tissue fragments. Modifications in isolated adipocytes relative to tissue fragments (or slices) have been demonstrated and, in particular, the method of using proteolytic enzymes for adipocyte isolation can also produce proteolytic changes at the cell surface such that the isolated cell is very dissimilar from its native state. In fact, it has been shown that rat cells produce more adenosine than slices,[40] and that PDE activity is lower in human and rat adipocytes than in tissue fragments.[41] Kather[42] has recently concluded that the adenosine that accumulates in adipocyte suspensions should be considered as a contaminant rather than as an endogenous regulator of fat cell function on the basis that it is almost exclusively derived from adenine nucleotides that are released by leaking cells. Such confounding effects would tend to produce artifacts biased toward a greater importance of adenosine in modulating various aspects of adipocyte metabolism. It is also difficult to elucidate the role of endogenous adenosine in modulating thermogenesis from studies that involve the addition of adenosine or its nonhydrolyzable analogues (eg, 2-chloro-adenosine). Whether added adenosine (or its analogue) has a minor or major inhibitory action on thermogenesis is very much dependent on the amount added. Indeed, we previously showed that addition of 2-chloro-adenosine suppresses the synergistic effect between a combination of ephedrine and caffeine on IBAT thermogenesis in a dose-dependent fashion.[16] However, by virtue of the fact that this approach involves addition of exogenous adenosine to the tissue preparation, such studies merely demonstrate the potent inhibitory effect of adenosine in excess of that present endogenously. Such an approach is hence unsuitable for assessing relative importance of endogenous adenosine antagonism in the mechanism by which caffeine and other xanthines interact with ephedrine to stimulate thermogenesis.

On the other hand, the main evidence against PDE inhibition as an important mode of action of methylxanthines rests on findings that the threshold concentration for PDE inhibition far exceeds their therapeutic plasma concentrations.[18,19] It is important to emphasize here that, on their own, dietary methylxanthines had no effect on IBAT MO_2 in the fed or fasted state, and that their mechanisms of action in our studies relate specifically to their interaction with ephedrine. In this context, the possibility therefore arises that in the presence of ephedrine (and hence increased NA release) subthreshold concentrations of methylxanthines as inhibitors of PDE activity are shifted toward much lower concentrations. This is supported by data presented here and previously[16] indicating that a similar threefold to fourfold increase in BAT MO_2 obtained by addition of caffeine (500 μmol/L) to ephedrine (0.1 μmol/L) could be achieved, as shown here, with lower concentrations of caffeine (50 to 100 μmol/L) but higher concentrations of ephedrine (0.25 μmol/L).

Finally, it is also possible that adenosine release/action and PDE activity may be altered in opposite directions in response to changes in sympathetic neural activity. Thus, decreased sympathetic nervous system activity (eg, with fasting) would result in an up- and down-regulation of PDE and adenosine, respectively; normal sympathetic nervous system activity (eg, normal feeding) would affect both feedback systems; and increased sympathetic nervous system tone (eg, during cold exposure) would result in down- and up-regulation of PDE and adenosine, respectively. This reciprocal interplay between adenosine and PDE, operating in the synaptic cleft and intracellularly, could serve to modulate functioning of the sympathetic nervous system for its role in thermoregulation and in the control of body energy stores. Such a hypothesis would also reconcile the discrepancy between data showing an important role for adenosine antagonism and none for PDE in the effect of theophylline in potentiating cold-induced thermogenesis,[43] with our findings here indicating that, under fasting conditions, only PDE inhibition but not adenosine antagonism is involved in the interaction between methylxanthines and ephedrine. Results of the present studies therefore reinforce the notion that, following caloric deprivation, an increase in PDE activity (rather than reductions in β-adrenoceptor number and sensitivity, reductions in adenylate cyclase activity, or increases in adenosine release and/or actions) plays an important role in diminished thermogenic and lipolytic responsiveness to catecholamines or sympathomimetic drugs.[44] Additionally, there is also evidence suggesting that PDE is critically involved in the antilipolytic effect of insulin in human fat cells,[45] an effect that would be enhanced due to increased insulin sensitivity following caloric restriction. Consequently, in the search for thermogenic drugs to assist in the management of obesity using low-calorie regimens, the targeting of PDE enzyme activities would seem to be a rational approach for enhancing both lipolytic and thermogenic effectiveness of sympathomimetics.

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