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Mitochondrial Oxidative Phosphorylation Controls SA-Induced Thermogenesis in the Appendix of *Sauromatum Guttatum*

Hanna Skubatz[1*]

[1]*NeoPro Labs, 3161 Elliott Ave, Suite 308, Seattle, WA 98121, United States of America.*

Author's contribution

The sole author designed, analyzed, interpreted and prepared the manuscript.

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ABSTRACT

Aim: To study thermogenesis induced by aspirin, salicylic acid and 2,6-dhdroxybenzoic acid in the appendix of the *Sauromatum guttatm* and *Arum italicum* inflorescences.
Study Design: Determination of tissue temperature in the presence of the three thermogenic inducers and mitochondrial inhibitors (antimycin, myxothiazol, salicylhydroxamic acid, 2,4-dinitrophenol, oligomycin and carboxyatractyloside).
Methods: Monitoring tissue temperature with thermocouples in the presence of inducers and different mitochondrial inhibitors over 48 h.
Results: Induced temperature rise was suppressed in the presence of different mitochondrial inhibitors. Inhibitors of complex III, Antimycin A (200 µM) and myxothiazol (200 µM), decreased significantly the rise in temperature independently of the inducer concentrations. Antimycin A (20 µM) combined with SHAM (2 mM), an alternative oxidase inhibitor, suppressed temperature rise. However, SHAM (2 mM) alone did not suppress the rise in temperature completely. An uncoupler, 2,4-DNP (1.5 mM) completely suppressed the rise in temperature. A diminished temperature rise was also detected in the presence of oligomycin (200 µM), an inhibitor of ATP synthase. Carboxyatractyloside, an adenine nucleotide translocator inhibitor, also suppressed the rise in temperature.

**Corresponding author: E-mail: skubatch@neoprolabs.com;*

Conclusions: These results indicate that the SA-induced thermogenesis is depending on the mitochondrial oxidative phosphorylation. It may also suggest the existence of a second source of heat in the mitochondria that is SHAM-insensitive.

Keywords: Arum italicum; aspirin; 2,6-dihydroxybenzoic acid; inducible thermogenesis; salicylic acid; Sauromatum guttatum.

1. INTRODUCTION

The prevailing view on thermogenesis in a mature appendix of the *Sauromatum guttatum* inflorescence is that overflow of electron through the mitochondrial electron transport chain is released as heat by the activity of the alternative oxidase (AOX). The expression of AOX is high at this stage of development [1]. It has been suggested that thermogenesis is regulated by several mechanisms: reduction of a disulfide bridge between AOX subunits [2], alpha-keto acids [3] and post-translational processing of AOX [4]. The importance of these activation mechanisms *in vivo is* unclear [5].

Three compounds induce thermogenesis in premature appendices (3-2 days prior to thermogenesis) of *Sauromatum* guttatum and *Arum italicum* [6]. The thermogenic inducers are aspirin (ASA), salicylic acid (SA), and 2,6-dihydroxybenzoic acid (2,6-DHBA). Both a cytochrome oxidase inhibitor (20 mM KCN) and an uncoupler of membrane potential (2.5 mM 2,4-dinitrophenol; 2,4-DNP) inhibit inducible thermogenesis [6]. These results are difficult to reconcile with the electron overflow theory because addition of KCN should increase electron flow via AOX [7], and the addition of 2,4-DNP should also increase electron flow via proton leakage [8].

The present paper provides evidence that mitochondrial oxidative phosphorylation (OXPHOS) activity is a prerequisite for the inducible thermogenesis in tissue slices of the *Sauromatum* and *Arum* appendices. Suppression of inducible thermogenesis was observed when *Sauromatum* appendix tissue slices were treated with a diverse set of mitochondrial inhibitors in the presence of the inducers. Two hundred µM antimycin A (AA) or 200 µM myxothiazol (Myx) significantly suppressed temperature rise. Both AA and Myx inhibit mitochondrial complex III CIII [9,10]. A combination of both inhibitors at a lower concentration (20 µM) resulted in a temperature peak with a broad shoulder that disappeared with increasing salicylhydroxamic acid (SHAM) concentration up to 2 mM. Suppression of temperature rise was also observed in the presence of 200 µM oligomycin (OM), a F_0F_1-ATP synthase inhibitor that prevents state-3 respiration [11]. Inhibition of the adenine nucleotide translocator ANT [12] by carboxyatractyloside (CATR) also suppressed temperature rise. ANT inhibits active mitochondria, between state-3 and -4 respiration [13]. The *Sauromatum* appendix tissue does not have any chloroplasts and therefore the inhibitors specifically affect mitochondrial activity. Similar suppression of temperature rise was observed in OM and 2,4-DNP treated tissue slices of the *Arum* appendix. These results suggest that under physiological conditions the temperature rise in *Sauromatum* and *Arum* appendices is under OXPHOS control.

2. MATERIALS AND METHODS

2.1 Reagents and Solutions

Chemicals were obtained from Sigma-Aldrich. Mitochondrial inhibitors (AA, CTAR, Myx, OM, and SHAM) were dissolved in either dimethyl sulfoxide or ethanol to make 20 mM stock solutions. 2,4-DNP was dissolved in distilled water to make 150 mM stock solutions. ASA, SA, and 2,6-DHBA were dissolved in distilled water to make 2 mM stock solutions.

2.2 Plant Materials and Growth Conditions

Corms of *Sauromatum guttatum* were kept at 4ºC and the inflorescences were allowed to develop under a 15/9 day/night cycle at ambient. When the inflorescence matures the base of the spathe becomes swollen and its color changes to burgundy. These characteristics appear at ~D-3 (3 d prior to D-Day, the day of inflorescence opening and thermogenesis). At this stage, a temperature rise can be induced by one of the inducers. *Arum italicum* was grown outdoors at the University of Washington, Seattle, USA. *Arum* inflorescences were cut at pre D-day stage, prior to spathe opening and thermogenesis.

2.3 Induction of a Thermogenic Response

Pre D-day appendices (D-3 to D-2) of the *Sauromatum* and *Arum* inflorescences were cut transversely into equal length slices (0.5 cm). The slices were placed in 24-well plates containing a thermogenic inducer solution plus inhibitor. The inducer solution consisted of one of the inducers, SA, ASA, or 2,6-DHBA in 0.5% Teeen-20 and 20 mM HEPES buffer, pH 7.0 [6]. A mitochondrial inhibitor was added to the inducer solution at the beginning of the experiment. The appendix slices were immersed in the solution throughout the experimental period in an environmental chamber (SANYO) under constant light regime at ~ 21ºC. Data collection started as soon as the appendix slices were placed in a solution.

2.4 Temperature Data Collection

Precision thermocouples (copper/constantan, Omega Engineering, Stamford, CT)) were inserted into the appendix slices and tissue temperature was recorded using a data logger (OMB- DAQ-56, Omega) every 2 min. There were 720 consecutive readings per 24 h. The effect of a treatment on the tissue temperature was calculated as the difference in temperature between a treated and a buffer treated tissue slice [6].

2.5 Data Analysis

Temperature rate was defined as ΔºC/h and was calculated by subtracting the first temperature reading at the first 2 min from the temperature reading at the first hour dividing by time. Temperature data smoothing to remove random variation was carried out with Excel's moving average.

3. RESULTS

3.1 Suppression of Induced Temperature Rise by Antimycin A and Myxothiazol in the *Sauromatum* Appendix

The effects of AA and Myx on temperature rise in tissue slices of the *Sauromatum* appendix were examined in an attempt to elucidate the role of the thermogenic inducers. A temperature rise above ambient with two distinct rates was observed (Fig. 1, row 1). Tissue temperature was strongly suppressed when treated with

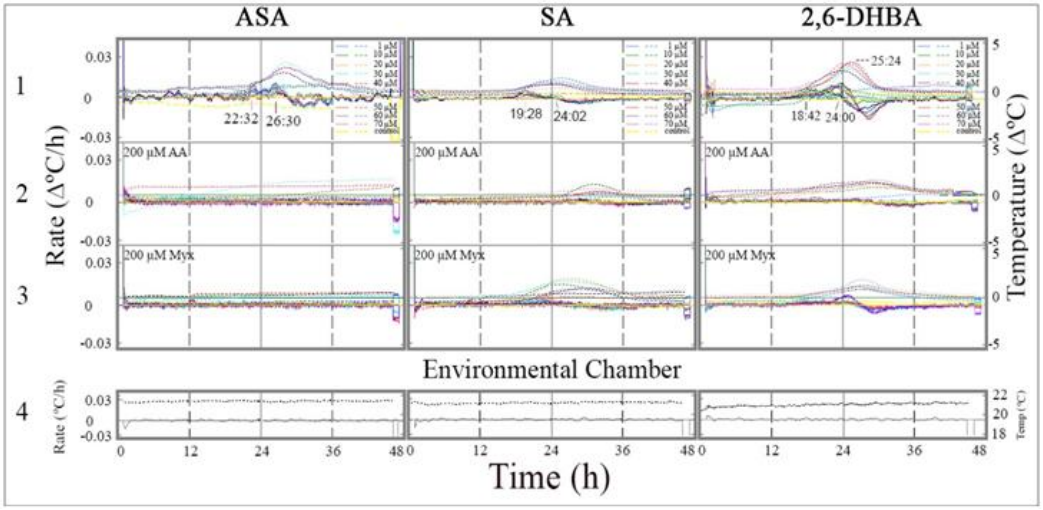

Fig. 1. Effect of antimycin A and myxothiazol on induced temperature rise in the *Sauromatum* appendix

Each column depicts tissue temperature in the present of one of the inducers (ASA, SA, and 2,6-DHBA) at increasing concentrations (1-70 µM). Row 1, tissue temperature in the presence of an inducer. Row 2, tissue temperature in the presence of inducer plus 200 µM AA. Row 3, tissue temperature in the presence of inducer plus 200 µM Myx. Row 4, temperature in the environmental chamber during experiments. Data are expressed as temperature values above chamber temperature (dashed lines, right Y axis) and corresponding rates (solid lines, left Y axis). The spike in temperature at the first h is temperature equilibration in the environmental chamber. Control (yellow line) is no treatment. Each treatment (one row) was carried out with two pre D-day appendices

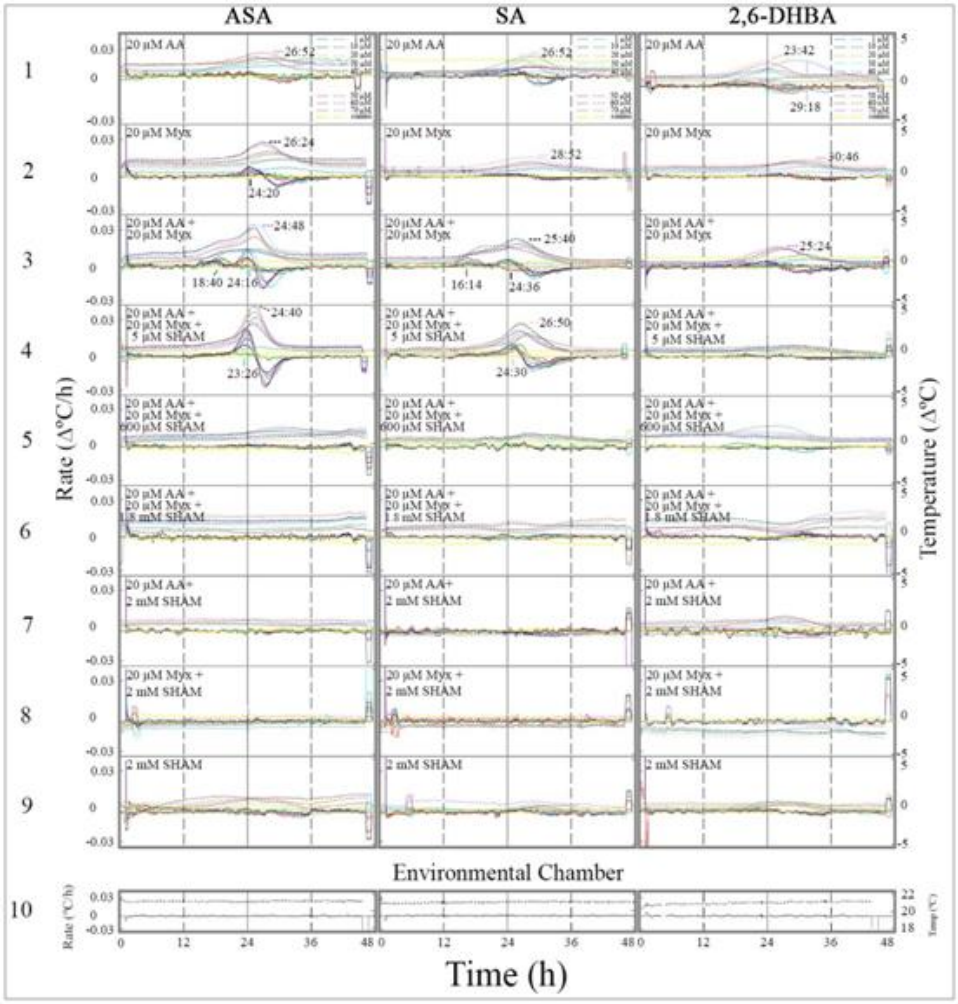

Fig. 2. Contribution of the alternative oxidase to induced temperature rise in the *Sauromatum* appendix

Tissue temperature treated with low concentration of AA (row 1) and Myx (row 2). Row 3, tissue temperature in the presence of low concentration of AA plus Myx. Rows 4-8, tissue temperature in the presence of low concentration of AA plus Myx and various concentrations of SHAM. Row 9, tissue temperature in the presence of high concentration of SHAM. Row 10, temperature in the environmental chamber during experiments

cytochrome III (CIII) inhibitors in combination with one inducer at a concentration range of 1-70 µM for 48 h: 200 µM AA (row 2) or 200 Myx (row 3). The suppression was independent of inducer concentrations. ASA-induced temperature rise was the most sensitive to CIII inhibitors. These results indicate that *in vivo* thermogenesis is under OXPHOS control. When CIII is completely inhibited thermogenesis does not occur and the electron flow from CI and CII is not diverted to AOX.

Partial inhibition of temperature rise in tissue slices was observed in the presence of 20 µM AA (Fig. 2, row 1), 20 µM Myx (row 2), or with both combined inhibitors (row 3). Treatment with 5 µM SHAM combined with 20 µM AA and Myx also caused a partial suppression of temperature rise in the presence of either ASA or SA (row 4). However, in the presence of 2,6-DHBA a complete suppression of temperature rise was observed. Treatments with higher concentrations of SHAM (0.6 mM - 2 mM) suppressed temperature rise in the presence of either ASA or SA (rows 5-7). Treatment with 20 µM Myx combined with 2 mM SHAM also suppressed temperature rise in the presence of ASA, SA, and 2,6-DHBA (row 8). Treatment with 2 mM

SHAM without any other inhibitors only partially suppressed the rise of temperature (row 9). It seems that temperature rise was more SHAM-sensitive in the presence of 20 µM AA than in its absence. It may suggest the presence of another heat generating system that is SHAM-insensitive and is sensitive to Complex III inhibitors.

3.2 Suppression of Induced Temperature Rise by Oligomycin in the *Sauromatum* Appendix

Treatment of tissue slices with 20 µM OM did not significantly affect temperature rise in the presence of all inducers (Fig. 3, row 1). However, treatment with 200 µM OM significantly suppressed temperature rise in the presence of ASA and, to a lesser extent, in the presence of SA (row 2). SHAM at 2 mM combined with 20 µM OM completely suppressed the rise in temperature in the presence of SA or 2,6-DHBA (row 3). Oligomycin prevents state-3 respiration and therefore it should facilitate electron flow via AOX. The suppression of temperature rise in the presence of SHAM combined with OM suggests that some of the temperature rise is the result of AOX activity. However, the partial suppression of temperature rise by OM may be an indication for AOX activity and a second energy-dissipating source are under OXPHOS control and are sensitive to ASA. The effect of OM on tissue slices treated with 2,6-DHBA was weak. Also, since the temperature did not rise in the presence of OM, a significant proton leak may not occur under these physiological conditions.

3.3 Suppression of Induced Temperature Rise by 2,4-DNP in the *Sauromatum* Appendix

The temperature of the appendix slices in the present of 150 µM 2,4-DNP did not rise significantly (Fig. 4, row 1). However, 1.5 mM 2,4-DNP completely suppressed temperature rise in the presence of all three inducers (row 2). It strongly suggests that mitochondrial membrane potential is required for temperature rise. 2,4-DNP combined with AA decreased temperature rise in the presence of ASA or SA but not in the presence of 2,6-DHBA (row 3). On the other hand, 2,4-DNP combined with SHAM decreased temperature rise in the presence of SA or 2,6-DHBA and, to a lesser extent, in the presence of ASA (row 4). It suggests that part of the temperature rise is a result of AOX activity and the rest of the temperature rise is generated by a second source. ASA strongly affects this second source of heat that is dependent on membrane potential.

3.4 Suppression of Induced Temperature Rise by Carboxyatractyloside in the *Sauromatum* Appendix

Carboxyatractyloside (CATR) binds to adenine nucleotide translocator (ANT) and competes with ADP for its binding site on ANT. It binds to ANT when mitochondrial respiration is between state 3 to state 4. Treatment of tissue slices with 200 µM CATR led to a significant decrease

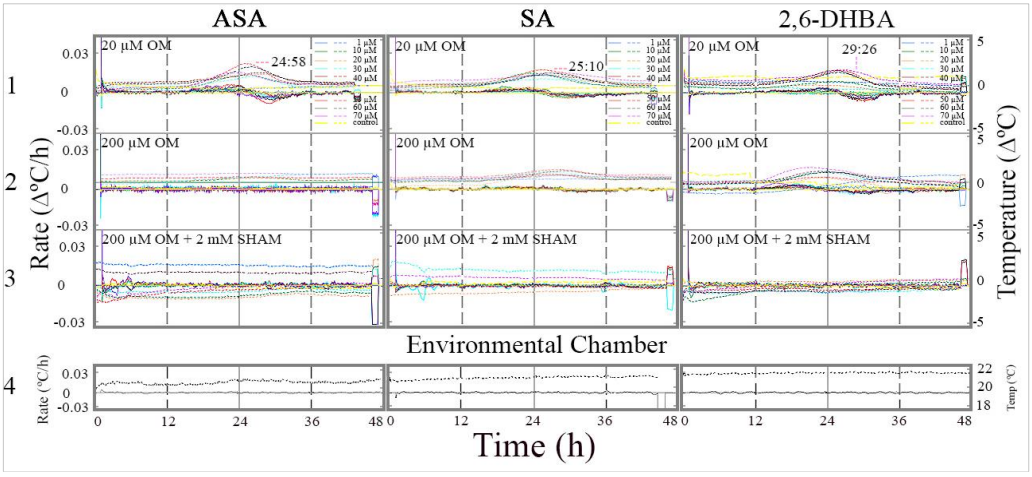

Fig. 3. Effect of oligomycin on induced temperature rise in the *Sauromatum* appendix

Row 1, tissue temperature in the presence of low concentration of OM. Row 2, tissue temperature in the presence of high concentration of OM. Row 3, tissue temperature in the presence of low concentration of OM plus 2 mM SHAM. Row 4, temperature in the environmental chamber during experiments

in temperature rise in the presence of ASA and 2,6-DHBA (Fig. 5, row 1). It again suggests that temperature rise is under OXPHOS control and ANT may be involved in this process.

3.5 Suppression of Induced Temperature Rise in the *Arum* Appendix

Temperature rise in tissue slices of premature *Arum* appendix treated with all inducers reached a peak at 7 to 9 h since the beginning of the experiment (Fig. 6, row 1). The temperature did not rise much in the presence of 200 AA but was not completely suppressed (row 2). Treatment with Myx combined with SHAM suppressed temperature rise in SA and 2,6-DHBA treated tissue slices but not in ASA treated slices (Row 3). Treatment with very high concentrations of SHAM (10 mM) did not suppress temperature rise completely (row 4). Treatment with OM suppressed temperature rise in the presence of ASA and SA (row 5). These results strongly suggest that the mechanism of temperature rise in the *Arum* appendix is similar to that of the *Sauromatum* appendix.

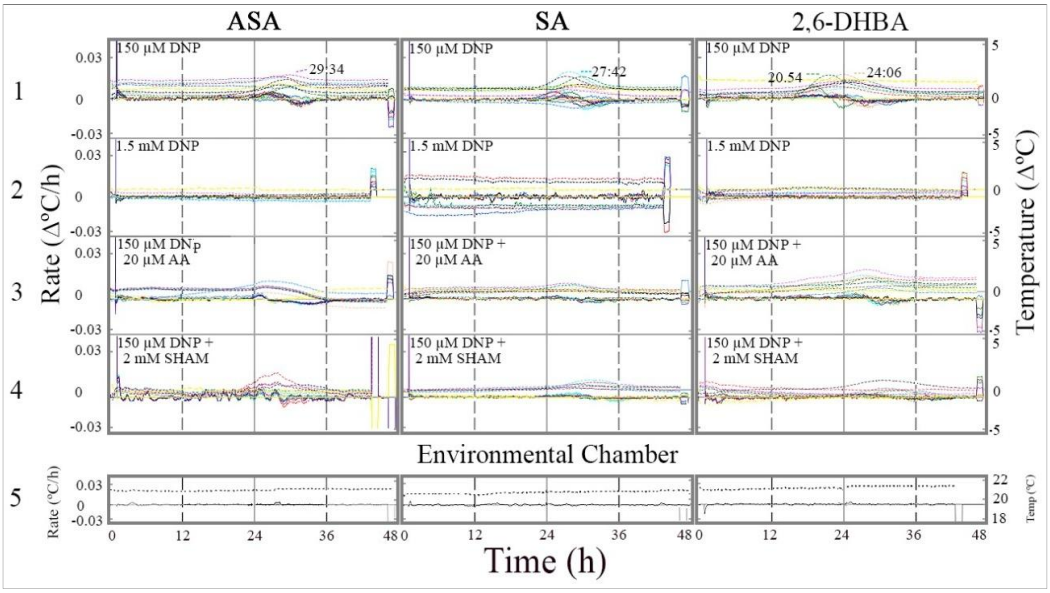

Fig. 4. Effect of 2,4-DNP on induced temperature rise in the *Sauromatum* appendix

Row 1, tissue temperature in the presence of low concentration of 2,4-DNP. Row 2, tissue temperature in the presence of high concentration of 2,4-DNP. Row 3, tissue temperature in the presence of low concentrations of 2,4-DNP plus AA. Row 4, tissue temperature in the presence of 2,4-DNP plus SHAM. Row 5, temperature in the environmental chamber during experiments.

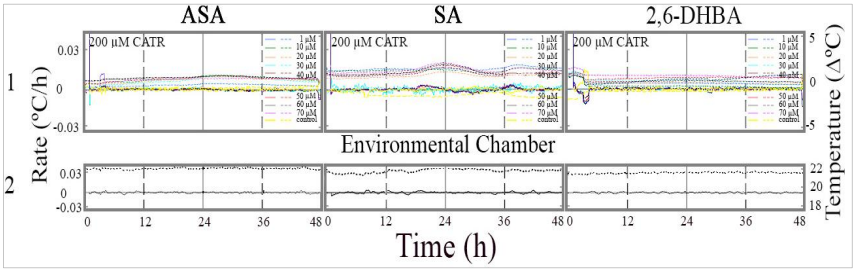

Fig. 5. Effect of carboxyatractyloside on induced temperature rise in the *Sauromatum* appendix

Row 1, tissue temperature in the presence of high concentration of CATR. Row 2, temperature in the environmental chamber during experiments

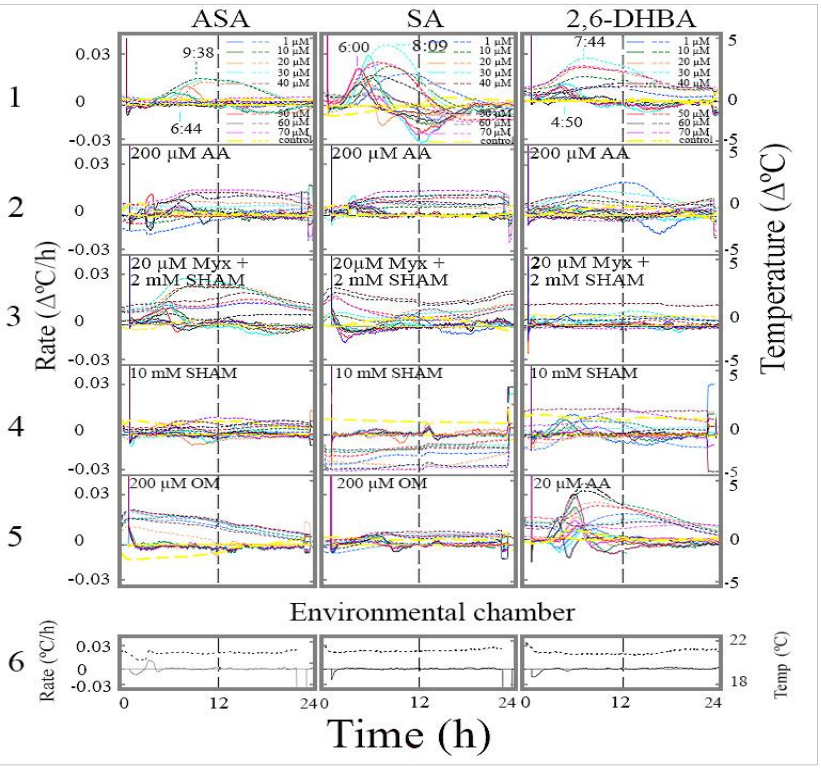

Fig. 6. Effect of mitochondrial inhibitors on induced temperature rise in the *Arum* appendix
Row 1, tissue temperature in the presence of various concentrations of inducers. Row 2, tissue temperature in the presence of high concentration of AA. Row 3, tissue temperature in the presence of low concentrations of Myx plus 2 mM SHAM. Rows 4, tissue temperature in the presence of high concentration of SHAM. Row 5, tissue temperature treated with high concentration of OM. Row 6, temperature in the environmental chamber during experiments

4. DISCUSSION

4.1 SA-Induced Thermogenesis is OXPHOS Dependent in the *Sauromatum* and *Arum* Appendices.

The binding of AA and Myx to CIII is non-competitive and irreversible with a 1:1 ratio [9, 10]. Treatment with both inhibitors completely inhibits electron transfer from ubiquinol to cytochrome b [14]. AOX is AA-insensitive and SHAM-sensitive because it interacts with a freely diffusible ubiquinone and bypasses the AA-sensitive CIII [5]. Treatment of leaf of *Arabidopsis thaliana* with AA and other mitochondrial inhibitors including OM increases the level of AOX mRNA [15]. Similar results were obtained in tobacco and petunia cells treated with CIII inhibitors over 48 h [16,17]. Therefore, it is unlikely that the treatment with AA and Myx decreased AOX level in *Sauromatum* or *Arum* appendices and consequently temperature did not rise. Even if treatment with AA and Myx caused a collapse of the proton gradient across the mitochondrial inner membrane, AOX should have remained active and the temperature should have risen.

Thermogenesis also requires the activity of F_1F_0-ATP synthase. Treatment with OM partially suppressed the rise of temperature. It has been shown that AOX1a mRNA was strongly induced by OM and weakly induced by 2,4-DNP [17]. Even the activity of ANT contributes to the level of thermogenesis. Together, these data suggest that the suppression of temperature rise is not a result of a decrease in protein expression but rather is dependent on functional OXPHOS.

4.2 Inducible Thermogenesis

4.2.1 Uncoupling proteins

Thermogenesis is based on uncoupling from oxidative phosphorylation. Uncoupling protein 1

(UCP1) is a mitochondrial inner membrane protein present in large amounts in brown adipose tissue of newborn and cold acclimated and hibernated mammals [18,19]. It mediates a proton leak across the mitochondrial inner membrane and causes non-shivering thermogenesis [20]. It is activated by free fatty acids and is inhibited by purine nucleotides [21]. UCP2 and UCP3 can mildly uncoupled OXPHOS [22]. In plants, coexistence of AOX and UCPs in mitochondrial inner membrane of plants has been demonstrated [23,24]. In thermogenic plants their expression depends whether the respiratory substrates is carbohydrates or lipids [2,8]. The temperature rise in the *Sauromatum* appendix is suppressed by full uncoupling and unaffected by mild uncoupling. This cannot be explained by AOX or UCP activities.

4.2.2 Uncoupling of adenine nucleotide translocator

ANT of plants is similar in structure and function to ANT of mammals [25]. Allosteric activation of the ANT can be involved in inducible proton leak [26,27]. For example, in brown-fat mitochondria, ANT1 mediates a significant part of the basal proton leak whereas ANT2 mediates fatty-acid-induced uncoupling [27]. The role of ANT in plant thermogenesis is not well studied.

4.2.3 Uncoupling of F_1F_0-ATP synthase

F_1F_0-ATP synthase can be transformed into an energy-dissipating enzyme under certain conditions [28,29]. The proton gradient can dissipate via proton back-leak through FO independently from the synthesis of ATP. Also, the c-subunit of this complex can be a leak channel sensitive to ATP/ADP ratio [30]. A relationship exists between F_1F_0-ATP synthase activity and the mitochondrial permeability transition pore (mPTP) opening. When mPTP is open ATP hydrolysis occurs, and it potentially can lead leading to the dissipation of the proton gradient and to thermogenesis.

4.2.4 Ca^{2+}-ATPase

Some studies suggest that Ca^{2+}-ATPase can indirectly dissipate energy in sarcoplasmic reticulum membrane of muscle tissue [31,32]. Futile cycling of calcium may lead to ATP hydrolysis and release of some heat [33]. Also, an increase in the rate of ATP hydrolysis can result in large amount of ADP changing ATP/ADP ratio that can stimulate OXPHOS and thereby increasing heat production.

5. CONCLUSION

The present study demonstrates the sensitivity to different mitochondrial inhibitors of induced temperature rise in the *Sauromatum* and *Arum* appendices. These results are in contrast to the widespread belief that most of the heat generated by AOX activity in these plants is not under OXPHOS control. Future studies are needed to identify the control of AOX activity in thermogenic tissues and the second source of heat.

COMPETING INTERESTS

Author has declared that no competing interests exist.

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