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Changes in Mitochondrial Respiratory Chain Components of Petunia Cells during Culture in the Presence of Antimycin A

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When petunia (*Petunia hybrida* Vilm, cv Rosy Morn) cells are cultured in the presence of 2 μM antimycin A (AA), respiration proceeds mainly via the cyanide-resistant pathway. Cyanide-resistant respiratory rates were higher in mitochondria from AA cells than in control mitochondria. Compared with control cells, an increase in alternative oxidase protein was observed in AA cells, as well as an increase in ubiquinone (UQ) content. A change in the kinetics of succinate dehydrogenase was observed: there was a much higher activity at high UQ reduction in mitochondria from AA cells compared with control mitochondria. No changes were found for external NADH dehydrogenase kinetics. In AA cells in vivo, UQ reduction was only slightly higher than in control cells, indicating that increased electron transport via the alternative pathway can prevent high UQ reduction levels. Moreover, O_2 consumption continues at a similar rate as in control cells, preventing O_2 danger. These adaptations to stress conditions, in which the cytochrome pathway is restricted, apparently require, in addition to an increase in alternative oxidase protein, a new setup of the relative amounts and/or kinetic parameters of all of the separate components of the respiratory network.

In most plants two pathways of electron transport from UQ to O_2 are present: the Cyt pathway and a CN-resistant, alternative pathway. It has been proposed that, in addition to a very specialized function in heat production in the Arum lily family, a more general function of AOX in the rest of the plant kingdom is that of a protector against oxidative damage as a result of stress situations. When the Cyt pathway is restricted, AOX engagement may prevent overreduction of respiratory chain components, in particular UQ, and subsequent generation of harmful reactive O_2 species (Purvis and Shewfelt, 1993; Purvis et al., 1995; Wagner, 1995). In addition, O_2 uptake continues so that the concentration will not increase to levels that favor the formation of reactive O_2 species (Wagner and Moore, 1997). This putative role is consistent with the observation that when stress situations are mimicked by inhibiting the Cyt pathway with AA, leading to high rates of production of superoxide anions and H_2O_2 in mitochondria (Boveris and Cadenas, 1982), *aox1* gene expression is induced and both AOX protein levels and respiratory capacity via the alternative pathway increase (Vanlerberghe and McIntosh, 1992, 1994; Wagner et al., 1992).

Petunia hybrida cells can survive, but do not grow, in the presence of 2 μM AA. Respiration in these cells proceeds mainly via the CN-resistant pathway: the addition of hydroxamates almost completely blocks respiration (Wagner et al., 1992), as has been observed in tobacco (*Nicotiana tabacum*) cells (Vanlerberghe and McIntosh, 1992, 1994).

The questions addressed in the present study were whether AOX activity indeed can maintain UQ reduction at control levels in petunia (*P. hybrida*) cells even when AA is present, and whether an increase in AOX protein in these mitochondria is the only adaptation to an inhibition of the Cyt pathway. Levels of the steady-state UQ reduction depend not only on the kinetics of the UQ-oxidizing pathways but also on the kinetics of dehydrogenases (Krab, 1995; Wagner and Krab, 1995); therefore, changes in these parameters can be expected to occur in addition to changes in the amounts of AOX protein.

To study these adaptations of electron transport chain components we used petunia batch cultures inoculated in culture medium with or without 2 μM AA. After 2 d of culture, cells were harvested, mitochondria were isolated, the amounts of AOX protein and Cyt *c* oxidase activity and the total amount of UQ were measured, and the relationships between rates and UQ reduction of oxidizing and reducing pathways in the mitochondria were determined. In addition, in vivo UQ reduction levels were measured.

MATERIALS AND METHODS

Petunia (*Petunia hybrida* Vilm, cv Rosy Morn) cell suspensions were grown in batch culture as described by Van Emmerik et al. (1992), in the absence or presence of 2 μM AA (Boehringer Mannheim).

Isolation of Mitochondria

P. hybrida mitochondria were isolated by homogenizing portions of 15 g fresh weight with a mortar and pestle at 4°C with 15 mL of a medium containing 0.4 M mannitol, 1 mM EDTA, 2 mM L-Cys, 0.2% BSA, 0.7% PVP-25, and 10 mM KPO_4 buffer, pH 7.4. The homogenate was pressed through a double layer of cheesecloth and centrifuged at 1000*g* for 5 min at 4°C. Centrifugation and washing steps were as described previously (Wagner, 1995).

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Abbreviations: AA, antimycin A; AOX, alternative oxidase; BHAM, benzohydroxamate; UQ, ubiquinone.

Measurements of Respiration

The respiration of cells and mitochondria was measured at 25°C using an O_2 monitor (model 53, Yellow Springs Instruments, Yellow Springs, OH) and a 5-mL (for cells) or 1-mL (for mitochondria) reaction vessel. Mitochondrial respiration was measured in a reaction medium containing 0.4 M mannitol, 10 mM KPO_4 buffer, 0.2% BSA, pH 7.1, with succinate (20 mM), NADH (2 mM), or a mixture of the two as substrates. To ensure complete activation of succinate dehydrogenase, 0.1 mM ATP was added. For state 3 measurements, 0.1 mM ADP was supplied. Respiration of cells was measured by transferring 5 mL of the cell suspension to the reaction vessel. The alternative pathway was inhibited with BHAM (2 and 13 mM for mitochondria and cells, respectively), and the Cyt pathway was inhibited with KCN (0.1 and 0.4 mM for mitochondria or cells). Mitochondrial protein was determined with the Bradford (1976) method.

Cyt c Oxidase Determinations

Cells were ground with a mortar and pestle first in liquid N_2 and, after thawing, in 0.1 M KPO_4 buffer, pH 7.2, and supplemented with 0.1% Triton X-100. Mitochondria were diluted 5-fold in 0.1 M KPO_4 buffer, pH 7.4, with 0.1% Triton X-100. Both preparations were centrifuged at 3000*g* for 10 min. All steps were performed at 4°C. The supernatants were used for the spectrophotometrical assay. Cyt *c* oxidase (EC 1.9.3.1) activity was measured at 550 nm in the presence of 0.03 mM reduced Cyt *c* in 10 mM KPO_4 buffer, pH 7.2. Cyt *c* (from horse heart, Boehringer Mannheim) was reduced with sodium dithionite; excess dithionite was removed by applying the Cyt *c*/sodium dithionite mixture to a Dowex column (type 1 × 8, mesh width 100–200 openings per inch) and eluting with distilled water. Activities (measured at 25°C) were calculated as the first-order rate constant *k* in grams fresh weight per minute (Van Emmerik et al., 1994).

UQ Extractions

Extraction and determination of reduced and oxidized UQ from isolated mitochondria and whole cells were performed as described prevoiusly (Wagner and Wagner, 1995). For each extract 0.5 to 1 mg of mitochondrial protein or 0.5 to 1 g of cells (fresh weight) was used.

SDS-PAGE and Immunoblotting

Up to 100 μg of mitochondrial protein was solubilized in sample buffer (312 mM Tris, pH 6.8, 10% [w/v] SDS, 10% [v/v] glycerol, 0.002% [w/v] bromphenol blue, and 100 mM DTT) and boiled for 1 to 2 min. The mitochondrial samples were subjected to SDS-PAGE (10% gel), followed by western analysis (Wagner, 1995). Antibodies developed against the AOX protein of *Sauromatum guttatum* (generously supplied by Dr. T. Elthon, University of Nebraska, Lincoln) were used at dilutions of 1:1000. Visualization was with a chemiluminescent reagent system (ECL, Amersham); 35-kD band intensities were scanned with a laser densitometer (2202 Ultrascan, LKB, Uppsala, Sweden).

RESULTS

AOX Protein Amounts and Cyt *c* Oxidase Activities

As observed before in tobacco (*Nicotiana tabacum*; Vanlerberghe and McIntosh, 1992, 1994), in *P. hybrida* cells the amount of AOX polypeptide was increased after culture in the presence of AA for 2 d. Scanning of the 35-kD bands (which is the form in which most of the *P. hybrida* AOX protein is present; Wagner, 1995) resulted in peak areas that were more than 3 times as large as in control mitochondria (Table I). Cyt *c* oxidase activities, on the contrary, were lower during culture with AA.

Total Amount of UQ

UQ was extracted from the mitochondrial membranes of petunia cells grown in the absence or presence of AA for 2 d. In AA cells twice as much UQ was extracted per milligram of mitochondrial protein as in mitochondria from control cells (Table I).

Kinetics of the Oxidizing Pathways

As reported previously (Wagner and Wagner, 1995), in petunia mitochondria a substantial portion of the total UQ pool is reduced in the absence of substrates (Fig. 1). This nonoxidizable pool was slightly higher in mitochondria isolated from AA cells than in control mitochondria (43% ± 6.5 and 55% ± 8.6, respectively; $n = 3$). The relationship between mitochondrial UQ reduction levels and uninhibited respiratory rates with NADH and/or succinate as the

Table I. *Amounts of AOX protein, Cyt c oxidase activities, and UQ concentrations in mitochondria isolated from petunia cells cultured for 2 d in the absence or presence of 2 μM AA*

Amounts of AOX protein were determined by scanning the 35-kD bands and are expressed as peak areas. Cyt *c* oxidase activities are calculated as the first-order rate constant *k*. Data are presented as mean values ± SD. Numbers in parentheses indicate the number of separate experiments.

Treatment	AOX Protein	Cyt *c* Oxidase Activity	UQ Concentration
	units 100 μg^{-1} protein	*k g^{-1} fresh wt min^{-1}*	*nmol mg^{-1} protein*
Control	0.14 ± 0.03 (4)	4.81 ± 0.89 (3)	0.48 ± 0.16 (12)
+2 μM AA	0.49 ± 0.03 (4)	3.23 ± 0.22 (3)	0.95 ± 0.17 (12)

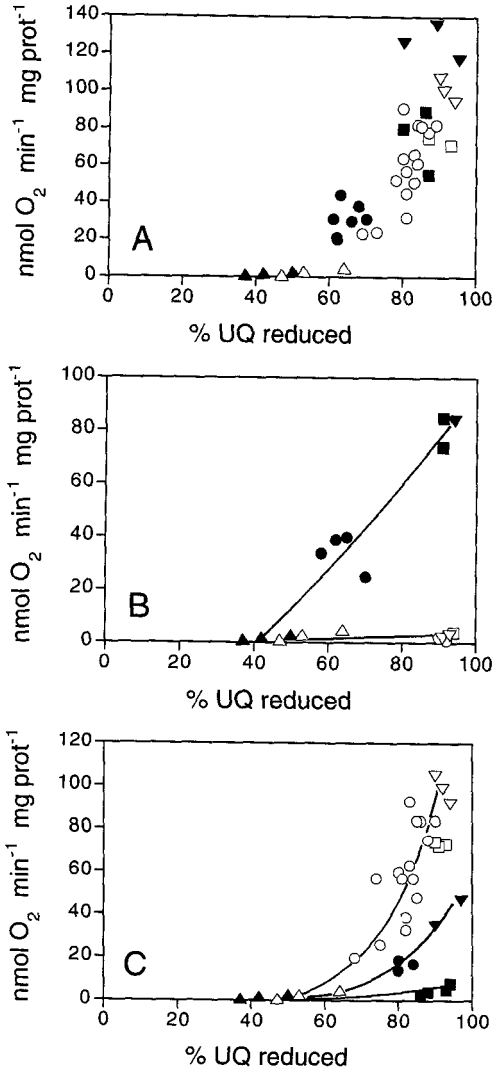

Figure 1. Relationships between rates and UQ reductions of uninhibited respiration (A) and Cyt- (measured in the presence of 2 mM BHAM) (B) and alternative pathway-mediated respiration (measured in the presence of 0.1 mM KCN) (C) in mitochondria from petunia cells grown for 2 d in the absence (closed symbols) or presence (open symbols) of 2 μM AA. Various steady states in the absence of substrates (△, ▲) or in the presence of 0.1 mM ADP with 20 mM succinate (±0–10 mM malonate, ○, ●), 2 mM NADH (□, ■), or a combination of succinate and NADH (▽, ▼) as the substrates are plotted. Data are from five separate experiments. prot, Protein.

substrate was not significantly influenced by the presence of 2 μM AA in the cell culture medium for 2 d (Fig. 1A).

After the addition of BHAM, however, it became clear that there was minimal O_2 uptake via the Cyt pathway in mitochondria isolated from AA cells (Fig. 1B), indicating that AA remains in the mitochondrial membranes during the isolation of the mitochondria. The maximal rate of CN-resistant respiration (with a combination of NADH and succinate) was higher in mitochondria from AA cells than in control cells (Fig. 1C). In control cells CN-resistant rates with NADH were very low (accompanied by a relatively high UQ reduction level), with succinate rates were higher, and with a combination of NADH and succinate the rates were twice as high as with succinate alone (due to the activation of AOX; Millar et al., 1993; Wagner et al., 1995). In contrast, in AA cells rates with NADH were almost as high as with succinate and were only slightly increased with the combination.

Whereas the data obtained with NADH as the substrate in the control mitochondria clearly could not be fitted with the same curve as the data with succinate and the combination of NADH and succinate, all data could be fitted with one curve in mitochondria from AA cells. The addition of pyruvate did not further increase rates with succinate or a combination of succinate and NADH in either control or AA mitochondria (data not shown).

Kinetics of Succinate and NADH Dehydrogenases

In addition to the kinetics of the UQ-oxidizing pathways, the kinetic properties of the UQ-reducing pathways are also important in determining the steady-state rates of mitochondrial respiration (Van den Bergen et al., 1994; Wagner and Krab, 1995). Since by definition in a steady-state situation the rate of UQ reduction equals the rate of UQ oxidation, it is possible to determine the rate of a UQ-reducing pathway by measuring the O_2 consumption in the steady state. In contrast to the determination of kinetics of the oxidizing pathways, in which the UQ-reducing side of the system is manipulated to obtain the various steady states, the kinetics of reducing pathways can be studied by manipulating the oxidizing side. One way to achieve this is by adding inhibitors of Cyt and the alternative pathway. By plotting the relationship between O_2 uptake rates with a certain substrate and UQ reduction levels at various steady states, the kinetics of the dehydrogenase in question can be determined (Van den Bergen et al., 1994; Wagner and Krab, 1995). Figure 2 shows such relationships between UQ reduction levels and rates of external NADH and succinate dehydrogenase in mitochondria isolated from petunia cells cultured for 2 d in the absence or presence of AA.

Whereas the external NADH dehydrogenase kinetics did not seem to have been affected by the AA treatment, succinate dehydrogenase kinetics were dramatically altered in mitochondria from AA cells. As observed before in potato tuber callus (Van den Bergen et al., 1994), relationships between rates and UQ reduction were very different for the two dehydrogenases in control mitochondria, with much higher rates obtained at high UQ reduction levels for the NADH dehydrogenase (Fig. 2, compare A and B). In AA cells however, succinate dehydrogenase exhibited kinetics similar to the NADH dehydrogenase.

In Vivo Respiration and UQ Reductions

Respiration and in vivo UQ reduction levels in whole petunia cells were measured as described previously (Wagner and Wagner, 1995). Total respiration was slightly higher in the presence of AA and was almost completely BHAM-sensitive (Fig. 3A). CN-resistant respiration was higher in AA cells than in control cells.

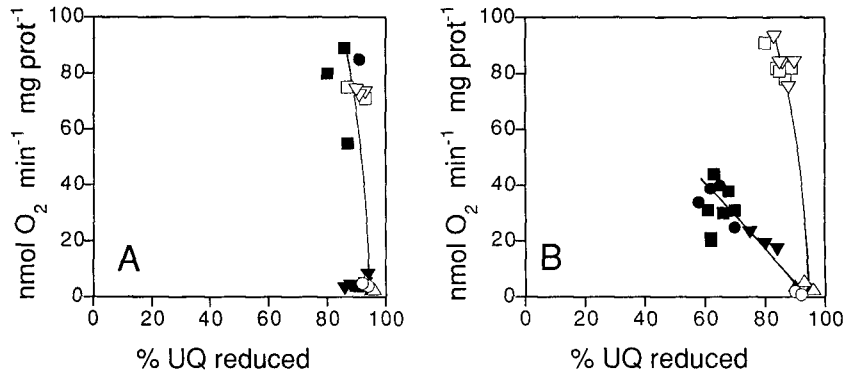

Figure 2. Relationships between rates and UQ reductions of external NADH dehydrogenase (A) and succinate dehydrogenase (B) in mitochondria from petunia cells grown for 2 d in the absence (closed symbols) or presence (open symbols) of 2 μM AA. Various steady states in the presence of 0.1 mM ADP with 20 mM succinate or 2 mM NADH as the substrate were obtained with no inhibitor present (□, ■); +2 mM BHAM (○, ●); +0.1 mM KCN (▽, ▼); or +2 mM BHAM and 0.1 mM KCN (△, ▲). Data are from five separate experiments. prot, Protein.

Although the Cyt pathway was clearly inoperative in cells after 2 d of culture in the presence of AA, in vivo UQ reduction was only slightly higher than that in the control cells (59% ± 3 versus 52% ± 5, $n = 5$; Fig. 3B). Whereas the addition of CN (or AA, data not shown) to control cells increased UQ reduction levels to the same levels reached when both CN and BHAM were given together, no effect on UQ reduction levels by CN was seen in AA cells. On the contrary, whereas the addition of BHAM did not have a significant effect on UQ reduction in control cells, it increased reduction levels to values higher than 80% in AA cells.

DISCUSSION

It has been postulated (Purvis and Shewfelt, 1993; Wagner, 1995; Wagner and Krab, 1995) that the major function of AOX in plant mitochondria is the protection against the overreduction of the respiratory components in situations in which the Cyt pathway is restricted, as reported for many stress situations. The addition of AA to plant cells mimicks such a stress situation. An increase in total AOX protein upon culture of petunia cells with AA was accompanied by higher CN-resistant respiratory rates in both mitochondria and cells. Whereas in tobacco cells (Vanlerberghe and McIntosh, 1994) the expression of Cyt *c* oxidase was unaffected by the presence of AA in the culture medium, in petunia cells Cyt *c* oxidase activities decreased upon culture with AA.

However, an increase in AOX protein appears not to be an isolated event, but is apparently part of a whole set of changes in the mitochondrial respiratory chain components. The kinetics of the AOX with NADH as the substrate is different in mitochondria isolated from AA cells compared with control mitochondria. Whereas the relationship between UQ reduction and the CN-resistant respiration rate shifts to the oxidized side when succinate (or malate or pyruvate; Millar et al., 1993, 1996; Wagner et al., 1995) is present in control mitochondria, the relationship between the CN-resistant rate and UQ reduction in AA mitochondria is not influenced by the presence of succinate (Fig. 2). One explanation may be that accumulated organic acids (e.g. pyruvate) in mitochondria from AA cells cause a permanent activation. Although no significant amounts of pyruvate could be detected in isolated mitochondria (data not shown), very low concentrations may be sufficient to cause this stimulation (Millar et al., 1996). On the other hand, the kinetics of the AOX for reduced UQ are also dependent on the reduction state of the regulatory disulfide bond (Umbach and Siedow, 1993), which may not be the same in control and AA mitochondria. The exact nature of the change in kinetics of NADH oxidation by the AOX during culture in the presence of AA will be the topic of further studies.

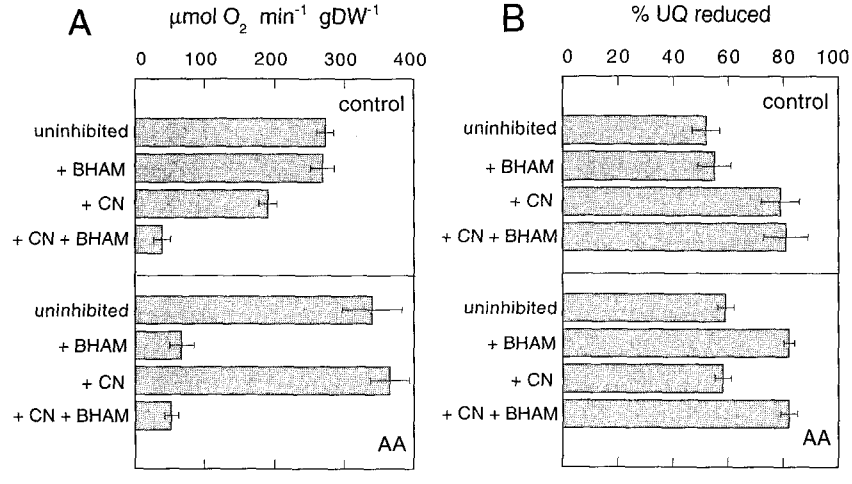

Figure 3. Whole cell respiratory rates (A) and in vivo UQ reduction levels (B) of petunia cells cultured for 2 d in the absence or presence of 2 μM AA. Rates were measured in the absence or presence of 13 mM BHAM and/or 0.4 mM KCN. Data are mean values from four separate experiments. Error bars represent SDs. DW, Dry weight.

In addition to these differences in the quinol-oxidizing pathway, the reducing side of the respiratory network is also affected. The kinetics of succinate dehydrogenase in the AA mitochondria allow much higher activity at high UQ reduction levels. At present, it is impossible to conclude from the data whether this change is caused by an increase in the total amount of the protein or by an increase in affinity. Another factor to take into account is the amount of substrate, in this case oxidized UQ, available for the enzyme. The total amount of UQ in mitochondria isolated from cells cultured in the presence of AA was twice as high as in control cells.

It has been suggested that the concentration of UQ can be a limiting component of electron transfer through the CN-resistant pathway (Ribas-Carbo et al., 1995). In a similar way, it might be argued that the increased UQ concentration in AA cells causes the different relationship between UQ reduction and O_2 consumption when succinate is the substrate in mitochondria isolated from these cells compared with control mitochondria. Modeling data, however, suggest that an increased UQ concentration does not necessarily lead to higher steady-state O_2 uptake rates and/or changes in the steady-state UQ reduction levels. This is because both UQ reduction by the dehydrogenases and UQ oxidation by the oxidizing pathways are reversible reactions, and an increase in UQ concentration will affect the rates in both directions (K. Krab, Vrÿe Universiteit Amsterdam, The Netherlands, unpublished data).

External NADH dehydrogenase kinetics were independent of the presence of AA in the culture medium, already indicating that an increase in the total amount of UQ available does not necessarily affect dehydrogenase rates and the relative amount of UQ reduced in the steady state. A similar conclusion can be drawn from the relative amount of UQ reduced in the absence of substrate. In spite of the increased amounts of total UQ in AA mitochondria, about 50% of the total UQ pool is still reduced in the absence of substrates.

Preliminary results suggest that an increase in AOX protein is not always accompanied by an increase in the total amount of UQ: after wounding of potato, AOX protein synthesis is induced, but mitochondrial UQ concentrations remain unchanged compared with fresh potatoes (A.M. Wagner and A.L. Moore, unpublished data). Therefore, the physiological role of the observed increase in UQ concentration when cells are cultured in the presence of AA is still unclear. An antioxidant role for UQ has been proposed (Landi et al., 1984), and, therefore, an increase in UQ concentration can be expected during conditions that promote the formation of reactive O_2 species. The relationship between UQ concentration and the kinetics of respiratory pathways, as well as the role of UQ as a protector against O_2 damage, will be subjects of further studies.

When the Cyt pathway is blocked by CN in control cells (Fig. 3), in vivo UQ reduction levels increase from ±52% to values higher than 80%. Such conditions are dangerous for the cell because of the potential production of reactive O_2 species that can react with the lipids in the mitochondrial membranes and therefore impair mitochondrial functioning (Purvis and Shewfelt, 1993; Purvis et al., 1995; Wagner and Krab, 1995). The normal output capacity of the alternative pathway is apparently not sufficient to maintain low reduction levels. After 2 d of culture in the presence of AA, however, in vivo reduction levels are not affected as a result of blocking the Cyt pathway. When the time course of the UQ reduction was followed after AA addition to the culture medium, it took about 15 h for the initially increased UQ reduction levels to reach the new steady state (data not shown).

Another important observation is that total respiration in AA cells is at least as high as in control cells, although O_2 uptake in the former proceeds mainly via the alternative pathway. Consequently, O_2 concentrations in these cells will be kept low, despite the inhibited Cyt pathway, pointing again to an important role of the AOX protein in controlling O_2 stress (Wagner and Moore, 1997).

In summary, it becomes clear from the present data that an increase in the amount of AOX protein is only one of a set of changes that takes place in plant cells cultured in the presence of AA. The process of adaptation to functioning with a restricted Cyt pathway requires a complete new setup of relative amounts and/or kinetic parameters of all of the separate components of the respiratory network to come to an adequate performance of the whole system under stress conditions.

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