Threshold Effect and Tissue Specificity
IMPLICATION FOR MITOCHONDRIAL CYTOPATHIES*

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Mitochondrial cytopathies present a tissue specificity characterized by the fact that even if a mitochondrial DNA mutation is present in all tissues, only some will be affected and induce a pathology. Several mechanisms have been proposed to explain this phenomenon such as the appearance of a sporadic mutation in a given stem cell during embryogenesis or mitotic segregation, giving different degrees of heteroplasmy in tissues. However, these mechanisms cannot be the only ones involved in tissue specificity. In this paper, we propose an additional mechanism contributing to tissue specificity. It is based on the metabolic expression of the defect in oxidative phosphorylation (OXPHOS) complexes that can present a biochemical threshold. The value of this threshold for a given OXPHOS complex can vary according to the tissue; thus different tissues will display different sensitivities to a defect in an OXPHOS complex. To verify this hypothesis and to illustrate the pathological consequences of the variation in biochemical thresholds, we studied their values for seven OXPHOS complexes in mitochondria isolated from five different rat tissues. Two types of behavior in the threshold curves can be distinguished corresponding to two modes of OXPHOS response to a deficiency. We propose a classification of tissues according to their type of OXPHOS response to a complex deficiency and therefore to their threshold values.

Mitochondrial pathologies are a heterogeneous group of metabolic disorders characterized by abnormalities of the mitochondrial ultrastructure as well as of oxidative phosphorylation functioning (1–4). During these last years, the study of mitochondrial DNA (mtDNA) has shown, in a certain number of cases, some precise mutation sites associated with a better clinical definition of the related pathologies (5–20). In addition, it has been shown that defects in oxidative phosphorylations (OXPHOS) are able to affect any tissue, thus leading to the concept of mitochondrial cytopathies (21). This underlies the problem of the variability of the phenotypic expression of an mtDNA mutation. Indeed, an OXPHOS deficit due to such a mutation will not necessarily lead to a pathology. Moreover, mitochondrial cytopathies present a tissue specificity characterized by the fact that even if a mutation is present in all tissues, only some will be affected, leading to the pathology (4, 22–25).

A first mechanism proposed to explain this tissue specificity is based on the random segregation of wild type and mutant mtDNAs during embryogenesis, giving different levels of heteroplasmy in tissues (4, 7, 26, 27). In this case, only tissues with a high proportion of mutated mtDNA would be affected. However, in patients where the mitochondrial mutation is homoplasmic (25, 28) and in the case of nuclear mutations giving a uniform deficiency in all tissues, this mechanism can no longer explain the tissue specificity. For this reason, we propose another mechanism based on the threshold effect in the expression of a defect. This effect can be characterized by the following: (i) a low quantity of normal mtDNA can suffice to maintain a normal level of oxidative phosphorylation, but (ii) a minute decrease in this quantity may make the respiration and ATP synthesis of mitochondria collapse.

This phenomenon has been particularly studied by Wallace and co-workers (4, 29, 30) who have shown that an mtDNA mutation could present a threshold effect at the level of the phenotypic expression. Since then, the same type of observation has been reported by various authors on different models (31–35). We have evidenced a more specific threshold effect concerning the expression of an OXPHOS deficiency on mitochondrial respiration (36–40), and we have shown that this biochemical threshold can be predicted in the framework of the metabolic control theory (41–43).

A mechanism that we propose in order to explain tissue specificity is based on this biochemical threshold effect. Indeed, its extent for each OXPHOS complex can vary according to the tissues, thus changing their sensitivity to a defect in this complex. To verify this hypothesis and to illustrate the pathological consequences of the variation in the biochemical thresholds, we studied their values for seven OXPHOS complexes in mitochondria isolated from five different tissues. Two types of behavior in the threshold curves can be distinguished corresponding to two modes of OXPHOS response to a deficiency evidencing either an “excess of enzyme activity” or a “buffering effect by the metabolic network.” We therefore propose a classification of the tissues according to the threshold values and the type of OXPHOS response to a given complex deficiency.

EXPERIMENTAL PROCEDURES

Chemicals
Rotenone, antimycin, oligomycin, carboxyatractyloside (CATR), a-cyano-4-hydroxycinnamate, cyanide (KCN), and N,N,N’,N’-tetramethyl-p-phenylenediamine (TMPD) were from Sigma.

Animals
Male Wistar rats weighing 200–300 g with free access to water and standard laboratory diet were used for this study. Experimental animals were sacrificed by cervical shock and decapitation.

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The abbreviations used are: OXPHOS, oxidative phosphorylations; CATR, carboxyatractyloside; KCN, cyanide; TMPD, N,N,N’,N’-tetramethyl-p-phenylenediamine.
Mitochondrial Preparations

Rat muscle (gastrocnemius, plantaris, and soleus) and heart mitochondria were isolated as detailed by Morgan-Hughes et al. (44).

Liver and kidney mitochondria were isolated as described by Johnson and Lardy (45).

Brain mitochondria were isolated from whole brain according to the method described by Clark and Nicklas (46).

Protein concentration was estimated by the Biuret method (47) using bovine serum albumin as standard. The mitochondria were made up to a concentration of 50–80 µg of protein per ml in their isolation buffer.

Oxidographic Measurements

Mitochondrial oxygen consumption was monitored at 30 °C in a 1-ml thermostatically controlled chamber equipped with a Clarke oxygen electrode, in the following respiration buffer: mannitol 75 mM, sucrose 25 mM, KCl 100 mM, Tris phosphate 10 mM, Tris/HCl 10 mM, pH 7.4, EDTA 50 µM plus respiratory substrates (pyruvate 10 mM in presence of malate 10 mM). The mitochondrial concentration used for this study was 1 mg/ml and the state 3 (according to Chance and Williams (48)) was obtained by addition of ADP 2 mM.

The respiratory rates were expressed in nanomoles of O/min/mg of proteins.

Enzymatic Determination

Complex I (NADH:Ubiquinone Reductase)—The oxidation of NADH by complex I was recorded using the ubiquinone analogue decylubiquinone as electron acceptor (49).

Complex III (Ubiquinol:Cytochrome c Reductase)—The oxidation of 20 µM ubiquinol by complex III was determined using cytochrome c (III) as electron acceptor (49).

Complex IV (Cytochrome c Oxidase)—Two methods for the determination of this step were used as follows: in the first, cytochrome c oxidase activity was determined spectrophotometrically using cytochrome c (II) as substrate (50). In the second, cytochrome c oxidase activity was measured polarographically in the presence of antimycin using ascorbate 3 mM and TMPD 0.25 mM as electron donor system.

All enzymatic activities were measured at 30 °C in a final volume of 1 ml and were expressed in micromoles of product formed per min and per mg of mitochondrial proteins.

Titration Curves

The titration curves of the various steps involved in the oxidative phosphorylations were determined using specific inhibitors of these steps: rotenone for complex I, antimycin for complex III, KCN for complex IV, oligomycin for ATP synthase, CATR for adenine nucleotide translocator, mersalyl for phosphate carrier, and a-cyano-4-hydroxycinnamate for pyruvate carrier.

The inhibition curves of the isolated mitochondrial respiratory rate were obtained at state 3 in the presence of pyruvate 10 mM, malate 10 mM, and enough ADP (2 mM) to maintain a stable steady state of respiration.

For complexes I, III, and IV, the inhibition curves of the respiratory rate (global flux) and of the enzymatic complexes (isolated steps) were determined experimentally. However, in some cases, it was impossible to determine the activity of the isolated step in the same conditions as for the global flux. This was the case for the ATP synthase, the adenine nucleotide translocator, the phosphate carrier, and the pyruvate carrier, the activity of which are dependent on the ΔψM generated by the respiratory chain. In these cases, the method presented by Gellerich et al. (51) was used. This method uses a non-linear regression that fits the respiratory rate inhibition curve. Non-linear fitting was done using the program Simfit (53).

For these complexes but also for complex IV, we drew, with the program TK Solver Plus (Universal Technical Systems, Rockford, IL), the inhibition curve of the isolated step activity (Fig. 1A, line b) using the parameters obtained by the fitting procedure and the model equations.

In a previous work (36), we had validated the use of this model by showing that for complex IV, the experimental and fitted titration curves were superimposable.

Threshold Curves and Determination of Threshold Value

The threshold curves come from the titration curves. Each point of a threshold curve represents the respiratory rate inhibition percentage as a function of inhibition percentage of the isolated step activity for the same inhibitor concentration.

For complexes I and III, the threshold curves were plotted graphically from the raw titration data. One point of the threshold curve represents the mean of several determinations on both titration curves (respiration on the ordinate versus isolated step on the abscissa) for the same inhibitor concentration.

For complex IV, ATP synthase, adenine nucleotide translocator, phosphate carrier, and pyruvate carrier, the threshold curves were obtained using the titration curves resulting from the fit of the respiratory rate titration curves (51).

The threshold values were determined as described in Villani and Attardi (52) with some modifications. Two linear regressions on the first and last points of the threshold curve were done using the least squares method. We then defined the threshold value as the abscissa of the intersection point between these two regression lines.

RESULTS

Titration Curves and Threshold Curves—In this paper, we use identical experimental conditions to study the variation in biochemical threshold for seven steps of the oxidative phosphorylations (complexes I, III, and IV of the respiratory chain, ATP synthase, adenine nucleotide translocator, phosphate carrier, and pyruvate carrier) in mitochondria isolated from five different tissues (muscle, heart, liver, kidney, and brain).

Determining this threshold effect necessitates the construction of the threshold curve. This requires the preliminary determination of experimental respiratory rate and isolated step activity titration curves. An example of titration curves is given in Fig. 1A, where the titration curve profile is different as follows: sigmoidal for respiratory rate inhibition (Fig. 1A, line a) and hyperbolic for isolated step activity inhibition (Fig. 1A, line b). This property, which can be explained in the framework of the metabolic control theory, makes it possible to understand the biochemical threshold effect observed in Fig. 1B (36, 37).

In addition, Fig. 1 shows that experimental and fitted titration curves (Fig. 1A) are superimposable as are the threshold curves (Fig. 1B), thus validating the fitting procedure (51, 53).

This fitting method has some advantages. It considers all points of the respiratory flux titration curve to calculate precisely the isolated step inhibition. Furthermore, using a fitting method is essential whenever it is experimentally impossible to determine the isolated step inhibition in the same conditions as for the global flux. This is the case for the activity of ATP synthase, adenine nucleotide translocator, phosphate carrier, and pyruvate carrier, which are dependent on the amount of ΔψM generated by the respiratory chain. Therefore, this method enabled us to construct threshold effect curves for these complexes.

However, although this fitting model is useful in this study, its application is nevertheless limited to non-competitive inhibitors and to sigmoidal respiratory rate titration curves. This is why it was impossible to fit the titration curves obtained with rotenone and antimycin (data not shown) which are not pure noncompetitive inhibitors (36). These latter two cases illustrate the limitations of the model.

In these cases (complex I and complex III), threshold curves were built graphically from the raw data (Fig. 2), whereas for the other complexes, we used data given by the fitting procedure (Fig. 3).

Threshold Curves Profiles—In Fig. 2 and Fig. 3, we can distinguish two types of threshold curves according to their profile.

Type I threshold curves present a plateau phase followed by a steep breakage allowing a precise determination of the threshold value. These types II are characterized by another curve where the breakage is no longer evident and where a precise threshold value is far more difficult to determine.

Type I threshold curves were observed for complex I and complex III (Fig. 2), whatever the tissue origin of mitochondria. The cytochrome c oxidase (Fig. 3A) presents the two profiles according to the tissue with a type I in the liver, the brain, and...
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Nevertheless, the threshold value is difficult to determine with precision in type II curves because their shape presents a less steep breakage than in type I curves, leading sometimes to the absence of a threshold effect (kidney/phosphate carrier). In this case, the choice of the points for linear regressions is more difficult. Thus, the threshold values of the type II curves will have less meaning and will be more qualitative than quantitative. In some cases, the visual comparison of the curves could be as much informative as the numerical values analysis (brain, liver, and muscle for the pyruvate carrier in Fig. 3).

Threshold Values in Different Tissues—For complex I, threshold values indicate a tissue difference (between 70 and 80% for the muscle, the liver, and the kidney versus 64% for the heart, and 50% for the brain), whereas for complex III all the values are high whatever the tissue (Table II).

As for complex III, threshold values for the adenine nucleotide translocator are high (approximately 85%) and do not present a large tissue variation.

However, it is possible to observe such a variation in the threshold value for the ATP synthase and the phosphate carrier (80% in the heart compared with 60% in the brain) and also for the complex IV which presents a high biochemical threshold in the liver, the kidney, and the brain (around 86%), and it is lower in the muscle and the heart (67%).

A tissue variation of the biochemical threshold is also observed in the case of the pyruvate carrier, with values around 70% in the liver and the muscle and 90% in the kidney.

Finally, note that all the threshold values we obtained are high (>50%) (Table II), whatever the complex studied and the tissue origin of the mitochondria. In other terms, for all the complexes it is necessary to have inhibited at least 50% of their activity before a decrease in at least 20% of the global flux (respiratory rate) is observed.

DISCUSSION

In this paper, we explain part of the tissue specificity observed in mitochondrial cytopathies by the existence of a threshold (37, 40) in the metabolic expression of a biochemical defect in an OXPHOS complex. We show that the threshold value for a given OXPHOS complex can vary according to the tissue. Furthermore, the threshold value can change according to the steady state of mitochondrial oxidative phosphorylations that can also vary in different tissues. Thus, if in a given tissue a step has a high biochemical threshold, a mutation giving a defect of this step will not necessarily lead to a significant decrease in the mitochondrial metabolism and consequently will not affect this tissue. Conversely, if in another tissue this same step has a low biochemical threshold, the same mutation could induce a pathology.

Control of Experimental Conditions—Many parameters such as the nature of mitochondria or the conditions of respiratory rate measurement (temperature, pH, and buffer composition) can change the respiratory steady state and therefore modify the threshold values (36, 37, 52). So that the observed threshold variations can be attributed to physiological tissue properties and not to variations in experimental conditions, it was important to control these conditions, both for mitochondrial isolation and respiratory rate measurement. Thus, only the nature of the mitochondria can be responsible for any variation detected in the biochemical threshold values between different tissues.

There is a risk of damaging mitochondria during their isolation and thus modifying the threshold. A loss of cytochrome c or an increase in the leak may lead to a new steady state and therefore to a possible modification of threshold values. A useful parameter to evaluate mitochondrial damage is the respiratory control ratio (48). Therefore, in our study, we used...
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FIG. 2. Complex I and complex III threshold curves. Percentage of respiratory rate as a function of percentage of complex I and III inhibition for rat mitochondria isolated from muscle, heart, liver, kidney, and brain. Each point comes from the titration curves and represents the percentage of the respiratory rate as a function of the percentage of the complex I activity for the same rotenone concentration. One point is the mean of the same rotenone concentration titration curves data.

mitochondrial preparations only when the respiratory control ratio was close to values reported in the literature for the same tissues and in the same conditions. Respiratory control ratio values routinely obtained are listed in Table I.

In addition, the choice of respiratory conditions is decisive so that only the nature of the mitochondria is responsible for the threshold value. Indeed, state 3 respiratory rate depends on experimental conditions and notably on the buffer composition. Since optimal respiratory buffers developed for mitochondria isolated from different tissues have too great a variation in their composition (phosphate concentration, isotony maintained by sucrose or KCl), we used the same respiratory buffer for all titration experiments.

Finally, the experimental parameter that we chose to characterize the steady state of the oxidative phosphorylations was the state 3 respiratory rate value. As shown in Table I, these values measured in mitochondria isolated from different tissues are similar, so the mitochondria in this experiment can be considered in the same steady state of respiration, whatever their tissue origin. The exception is the brain where the state 3 respiratory rate has an inferior value.

Explanation of Threshold Curve Profiles—Threshold curves obtained in this study are similar to those already observed by many authors (37, 39, 52, 54–60). In Figs. 2 and 3, however, two profiles can be distinguished independently of the step studied and of the mitochondrial tissue origin.

Threshold curve profiles can be explained by an excess of enzyme activity that accounts for the plateau phase of type I threshold curves. This excess of enzyme activity can be due to an excess of enzyme (excess protein) or to up-regulating the intrinsic activity by modification in the apparent kinetic properties (different apparent $K_{m}$ values for instance) of the enzymatic complex. On the other hand, if there is no excess of enzyme activity, the isolated step inhibition will have a direct effect on the flux (type II profile), so the threshold curve will no longer have a plateau phase.

However, a second mechanism can be implied in the type I and type II threshold curve profiles. This mechanism can be explained in the framework of metabolic control analysis (41–43, 61) and involves the buffering of individual step perturbation in a metabolic network. According to this theory, the metabolic network (kinetic properties of the enzymes and structure of the system, intermediary pools of substrates, etc.) is responsible for this buffering effect. On threshold curves, buffering will give a more or less pronounced breakage. This effect is not important for a precise determination of the threshold value for type I curves because this value is principally dependent on the length of the plateau phase. Conversely, for type II curves, the buffering effect is largely responsible for the smooth shape of the curve and therefore of the difficulty in the determination of the threshold value.

In summary, type I threshold curves should correspond to enzymes with a high excess of enzyme activity and type II threshold curves to enzymes with a low one. For a given enzyme, whatever its excess of activity, the buffering effect by the metabolic network will be involved in the threshold effect and will be more decisive for giving the shape of type II curves.

A good example to illustrate the excess of enzyme activity and its role is shown in the threshold curve of adenine nucleotide translocator in liver mitochondria respiring on different substrates (Fig. 3F). Indeed, for this carrier, the following two profile types can be observed when the mitochondrial respiration is modified by changing respiratory substrates: type I on pyruvate and type II on succinate. This phenomenon can be explained by the fact that on succinate the oxygen consumption flux (respiratory rate) increases (211 ± 13 nanoatoms of O/min/mg of proteins against 142 ± 20 nanoatoms of O/min/mg of proteins on pyruvate) and that the amount of adenine nucleotide translocator 290 pmol/mg proteins (estimated by the quantity of CATR that completely inhibits phosphorylation) allows an excess of enzyme activity on pyruvate respiration but not on succinate. Thus, the threshold value obtained with pyruvate (92 ± 1.6) was considerably decreased when succinate was used (58.6 ± 6.62). Conversely, in muscle, there is no type II threshold curve on succinate because the higher amount of adenine nucleotide translocator (1600 pmol/mg proteins) allows an excess of enzyme activity on pyruvate and even on succinate (see also Doussiére et al. (58)). This behavior stresses the fact that excess of enzyme activity depends upon the OXPHOS steady state, which is in turn determined upstream by
the respiratory substrate and downstream by the energy demand of the tissue (30). In summary, all the threshold curves obtained in this study clearly show that the two profile types (type I and II) can be observed for some complexes depending on the tissue origin of the mitochondria and the OXPHOS steady state.

Threshold Values in Different Tissues and Tissue Groups—
For complex III and the adenine nucleotide translocator, threshold values are high and do not present a tissue variation. Thus, for these two complexes, there could be a large excess of enzyme activity in all tissues. For complex I, the length of the plateau on the threshold curves is in general shorter and presents a tissue variation. This leads to weaker threshold values indicating a tissue difference (around 70–80% for the muscle, the liver, and the kidney versus 64% for the heart and 50% for the brain).

For complex I, this tissue variation could in part be due to different excess of enzyme activity that could be correlated to variations in the amount of complex and/or to the presence of isoforms of nuclear origin differing in their regulatory properties.

All the phosphate carrier, ATP synthase, and pyruvate carrier threshold curves (Fig. 3) present a type II profile (except for the pyruvate carrier in kidney). This difference with complexes I, III, and the adenine nucleotide translocator cannot be explained by an excess of enzyme activity but has to be interpreted in terms of buffering effect by the metabolic network. Indeed, the different threshold values could be related to the different buffering capacities of the tissues.

In Table II, it also appears that threshold values for these complexes (from 60 to 79%) are weaker than those for complex III and for the adenine nucleotide translocator (from 80 to 90%).

For complex IV, the threshold curves present the following two types of profiles according to the tissue considered (Fig. 3A): type I for the liver, kidney, and brain and type II for the muscle and heart. This observation could be once again linked to an excess of enzyme because the spectrophotometric quan-
The variation in biochemical threshold values for a machinery. These tissues and therefore to the nature of their mitochondria, characterize either one or the other of these two groups according to the complex. For instance, in our experimental conditions, threshold value analysis for the different tissues shows that for complex IV in the muscle and heart, the threshold values (approximately 67%) are lower than in the kidney and brain (approximately 86%). In this case, the liver can be associated with the kidney and the brain.

These two groups of tissues can also be observed in the phosphorylations for ATP synthase and phosphate carrier (approximately 78% for the muscle and the heart versus approximately 66% for the kidney and the brain). The threshold value for the liver stays in between so that it is difficult to associate this tissue with one or the other group.

This property could be related to the energy metabolism of these tissues and therefore to the nature of their mitochondria, and more particularly to the balance between the amount of respiratory enzymatic complexes and the ATP synthesis machinery.

Threshold and Tissue Specificity in Mitochondrial Cytopathies—The variation in biochemical threshold values for a given complex according to the tissue origin of the mitochondria suggests a mechanism to explain the phenomenon of tissue specificity observed in mitochondrial cytopathies. For a given OXPHOS complex, the lower the threshold value in a tissue, the more sensitive this tissue to a defect of this complex. For example, Fig. 3 shows that if a mutation in cytochrome c oxidase leads to an 80% decrease in its activity, it will induce a smaller decrease in mitochondrial respiration in liver, whereas the respiration will be decreased to 40% in heart.

In addition, note that all the threshold values we have obtained are high (>50%). This phenomenon could be a way to provide a safety margin for oxidative phosphorylation against a defect in one or several of its complexes. This observation could be correlated with the fact that in most patients with clinical features of mitochondrial cytopathies of mtDNA mutation origin, the proportion of mutant mitochondrial DNA almost always exceeds 50% (25).

Nevertheless, the variation in the biochemical threshold values is not the sole mechanism involved in tissue specificity. Other mechanisms can intervene upstream from the biochemical threshold effect. This is notably the case of a specific mutation in a given stem cell during embryogenesis (62) or the mitotic segregation and the degree of heteroplasmy that are responsible for the different levels of an enzymatic deficit in tissues. In all cases, the biochemical threshold effect will play a role in the repercussion of this enzymatic deficit on respiratory flux. In fact, the coexistence of these mechanisms can be responsible for tissue specificity in mitochondrial pathologies.

Finally, it should be pointed out that this study was done on isolated mitochondria, in conditions far from in vivo ones. Indeed, in our experimental conditions the mitochondria were in the same steady state of respiration. However, in vivo, the tissues are in different steady states, and this can play an additional role in the distribution of threshold values, irrespective of the nature of the mitochondria. In vivo, these two parameters, i.e., nature of the mitochondria and the metabolic steady state of the tissue, might both affect the distribution of the threshold value in the tissue. Thus, although our results cannot be directly applied to mitochondrial cytopathies, they show how the biochemical threshold effect is a basic mechanism in the phenomenon of tissue specificity in oxidative phosphorylations.

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