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Review

Mitochondria and ischemia–reperfusion injury of the heart: Fixing a hole

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Abstract

Ischemia and post-ischemic reperfusion cause a wide array of functional and structural alterations of mitochondria. Although mitochondrial impairment is recognized as pivotal in determining loss of viability, the causal relationships among the various processes involved is ill defined. Nevertheless, a wide consensus exists in attributing a crucial role to opening of the mitochondrial permeability transition pore (PTP). Strong support for this concept has recently been provided by the reduced infarct size observed in mice lacking cyclophilin D. This protein located within the mitochondrial matrix favours PTP opening by increasing its sensitivity to Ca^{2+} in a process that is antagonized by cyclosporin A. Genetic approaches have also been used to demonstrate that adenine nucleotide translocase is not an essential component of the PTP. Here, we discuss our current understanding of the structure and function of PTP in the context of heart injury caused by ischemia and reperfusion.

Keywords: Mitochondria; Permeability transition; Reactive oxygen species; Ischemia; Cell death

1. Introduction

Mitochondrial dysfunction affects cell viability through a wide array of events. Loss of ATP synthesis and increase of ATP hydrolysis, impairment in ionic homeostasis, (Ca^{2+} in particular), formation of reactive oxygen species (ROS) and release of proapoptotic proteins are all recognized as key factors in the generation of irreversible damage [1–5]. This series of events explains why mitochondria are involved in both necrosis and apoptosis following post-ischemic reperfusion. Despite the general consensus on the role of mitochondria in cell death, relevant questions remain unsolved, especially concerning molecular mechanisms and causal relationships. The study of underlying mechanisms is made difficult by the fast rate with which irreversible injury occurs during post-ischemic reperfusion, and is often clouded rather than clarified by results derived from drugs that lack the selectivity needed for reliable biochemical insights.

In recent years great interest has been devoted to the mitochondrial permeability transition pore (PTP) (reviewed in [6–10]). Its role in the reperfusion injury of the heart was hypothesized at the end of the 80s [11,12], and subsequently demonstrated in isolated cardiomyocytes [13] and in perfused hearts [14]. PTP opening has also been proposed to play a role in both ischemic preconditioning (IPC) and postconditioning [15–17]. Mitochondria indeed appear to play relevant roles in endogenous mechanisms of protection as well [18]. For instance, it has been shown that a slight increase in ROS formation is associated with boosting of self-defense mechanisms [19,20]. Indeed, antioxidants abrogate the powerful protection afforded by IPC [20]. It has been proposed that mitochondria are involved in this protective mechanism(s) through the opening of K_{ATP} channels [21–23] and/or of the permeability transition pore (PTP) [24].

In the present review we discuss recent advances in PTP structure and function with the aim of assessing its role in ischemia–reperfusion injury of the heart.

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2. Functional and structural aspects of the permeability transition pore

2.1. The permeability transition pore (PTP)

The PTP is a voltage-dependent, high-conductance channel located in the inner mitochondrial membrane (IMM). In the fully open state, the apparent pore diameter is 3 nm, allowing passive diffusion of solutes with molecular masses up to about 1.5 kDa ([25,26], and references therein). A relevant feature of the PTP is its inhibition by cyclosporin A (CsA). Since the effect of CsA can be relieved by increasing the Ca^{2+} load [26], the effect of CsA is best described as "desensitization" of the PTP to Ca^{2+}. This is a key point because the PTP can still open in the presence of CsA, a fact that demands careful controls in order to assess whether the PTP is actually inhibited after administration of CsA in vitro and in vivo [27].

Because of their masses larger than the PTP exclusion limit, proteins do not diffuse through the pore. Consequently, when mitochondria are suspended in a crystalloid buffer, matrix proteins exert a colloidosmotic pressure that causes mitochondria to swell. Even a large increase in matrix volume does not damage the IMM, which is protected by cristae unfolding. Indeed, pore closure in saline media is followed by inner membrane refolding and full recovery of function, provided that cytochrome *c* is added back [28]. On the other hand, matrix swelling may cause the rupture of the outer membrane (OMM) and release of proteins from the intermembrane space. An important member of this group is cytochrome *c*, which after binding Apaf-1 in the cytosol causes the activation of caspase 9, triggering the apoptotic cascade [5,29]. Notably, when the matrix volume is normal, the largest fraction of cytochrome *c* is sequestered within IMM cristae [30]. Therefore, in the absence of matrix expansion, only a minor fraction of cytochrome *c* is available for its release through the OMM through pores formed by proapoptotic members of the Bcl-2 family [31,32]. This notion suggests that PTP is also relevant for the intramitochondrial redistribution of cytochrome *c* in cells undergoing apoptosis when the entire pool of this chromoprotein is released from mitochondria.

Mitochondrial swelling is generally considered synonymous of PTP opening. However, it must be pointed out that (i) swelling might not occur in intact cells due to the high cytosolic content of proteins and macromolecules [26] . For instance, PTP-induced swelling in vitro is abolished when mitochondria are incubated in the presence of solutes of proper size, such as 3.4 kDa polyethylene glycol [33]; (ii) very short open times and/or lower conductance states of PTP might not cause detectable changes in matrix volume [34,35]. Indeed, different PTP opening states have been demonstrated in isolated mitochondria and in intact cells. A subconductance state was initially suggested by showing that mitochondria impermeable to sucrose could still release Ca^{2+} in a process that occurred in the absence of added Na^{+} and was not inhibited by ruthenium red [36]. These findings also indicate that the PTP is independent of Na^{+}/Ca^{2+} exchanger and Ca^{2+} uniporter. Subsequently, electrophysiological measurements of isolated mitochondria demonstrated that, besides the conductance of 1200 pS of the PTP in its fully open state, a half conductance of 500 pS can be detected [25]. Such a lower conductance would correspond to an exclusion size of <300 Da allowing ion diffusion [34]. It has been proposed that the pore in this conformation should flicker allowing the release of accumulated Ca^{2+} in a pH-dependent process favoured by matrix alkalinization [37]. The efflux of Ca^{2+} by means of PTP opening would be advantageous since the consequent depolarization would prevent an immediate reuptake of the released Ca^{2+} [38]. The function of PTP as a Ca^{2+} release channel might explain the increase in calcium detected in mitochondria isolated from CsA-treated cardiomyocytes [39]. In addition, the results of elegant experiments performed in isolated cells suggested the involvement of PTP in amplification of the Ca^{2+} signals primarily originating from the endoplasmic reticulum [34,40].

Irrespective of its conductance, PTP opening is reversible. Transient openings were demonstrated by monitoring the redistribution of calcein [41] (the use of fluorescent probes in mitochondrial studies was reviewed in [42]). However, due to its molecular mass (622 Da) the intracellular redistribution of calcein does not reflect PTP subconductance states in situ, at variance with the interpretation given by a recent report on PTP occurrence and role in preconditioning [24].

Our studies on the relationship between opening of the PTP, mitochondrial depolarization, cytochrome *c* release and occurrence of cell death indicate that CsA-inhibitable release of cytochrome *c* and cell death correlate with the changes of mitochondrial TMRM fluorescence (which reports the mitochondrial transmembrane potential) but not with those of calcein fluorescence (which reports PTP opening). Since pore opening must be accompanied by depolarization, we concluded that short PTP openings are detected only by trapped calcein and may have little impact on cell viability; while changes of TMRM distribution require longer PTP openings, which cause release of cytochrome *c* and may result in cell death [43]. Therefore, duration of PTP opening appears to be a key element in determining the outcome of stimuli that impinge on mitochondria.

2.2. Consequences of PTP opening

Besides increasing matrix volume, PTP opening results in major modifications of mitochondrial function and structure that eventually jeopardize the maintenance of cell viability. The immediate consequence of PTP opening is the collapse of mitochondrial membrane potential ($\Delta\psi_m$). As a consequence oxygen consumption is initially increased and ATP produced by glycolysis is hydrolyzed by the reverse

operation of F_oF_1 ATPase leading to ATP depletion (reviewed in [7,9,10]). These initial events can be followed by additional alterations making the outcome quite complex and hard to predict. As discussed below, PTP opening allows the efflux and then the hydrolysis of pyridine nucleotides [44] resulting in a decrease of oxidative metabolism and oxygen consumption. In addition, oxidative stress might be exacerbated, since NAD(P)H is crucial for the maintenance of both mitochondrial and cytosolic antioxidant defenses. On the other hand, the increase of Mg^{2+}, H^+ and ADP concentration might limit the duration of PTP opening, or its spreading to a significant number of mitochondria in a given cell.

It is worth pointing out that although $\Delta\psi_m$ is inevitably collapsed by PTP opening, the reverse is not always true. Indeed, PTP opening does not always occur in cells treated with the mitochondrial uncoupler FCCP [43,45]. Therefore, a given process cannot be attributed to PTP simply based on the $\Delta\psi_m$ changes.

These functional consequences of PTP opening are associated with a wide array of structural changes of mitochondria. Besides cristae remodelling and outer membrane alterations discussed in Section 2.1, it has been proposed that structural changes caused by PTP opening might prompt the removal of damaged mitochondria by means of autophagy [46,47]. This process of "mitoptosis" could also be triggered by other processes acting at the level of the outer mitochondrial membrane independently of PTP opening [48].

2.3. Molecular identity of PTP: an unsolved riddle

The molecular identity of PTP has not been elucidated yet. This represents a major hurdle not only in the advancement of our knowledge on this relevant process, but also in the development of specific inhibitors. PTP has been suggested to be formed by the interaction of several proteins that would connect the mitochondrial matrix to the cytosolic space. The minimum set would be constituted by assembling cyclophilin D (CyP-D) in the matrix with adenine nucleotide translocase (ANT) in the IMM, which would then connect to VDAC in the OMM (the arguments supporting this hypothesis have been reviewed in [10]). This relatively simple scheme has been made more complex by adding several other proteins in order to accommodate the bewildering variety of effectors acting on the PTP [49,50]. These models are partly based on the effects of drugs and partly on experiments where proteins isolated and reconstituted in artificial membranes were tested for properties expected of the PTP [49,51–53]. Based on these experiments, it has been proposed that the ANT is the essential core component that would be converted into PTP by interacting with CyP-D. This interaction would be modulated by CyP-D ligands, such as CsA, or ANT ligands, such adenine nucleotides or ANT inhibitors. This interpretation accommodates many observations on PTP regulation, but fails to explain the control exerted by molecules that do not interact with ANT, such as quinones [54].

The requirement for ANT as an essential molecule in the formation of a functional PTP has been seriously undermined by the results obtained in mice lacking ANT [55]. The two isoforms of this translocator were genetically inactivated in mouse liver, and their absence was confirmed by immunoblot analyses and functional studies indicating the absence of ADP-stimulated respiration (i.e., state 3). Nevertheless, a CsA-inhibitable PTP could still be detected, although it required a larger Ca^{2+} load. Therefore, ANT might play a regulatory role, but (i) it is not an essential component of the PTP, and (ii) it is not the receptor for CyP-D. Although the interpretation of these experiments appears quite straightforward [55], it has been argued that it is still open to question because a low level of ANT expression could have been present in the liver, producing the PTP observed in ANT-null mitochondria [56]. The data of Kokoszka and Wallace, however, document that this conjecture is untenable because the PTP in ANT-null mitochondria was insensitive to opening by atractylate and to closure by ADP (compounds that affect the PTP through the ANT). It is very difficult to see how these ANT molecules would not respond to atractylate and ADP, and yet be able to promote a CsA-sensitive PT.

A genetic approach was also used for elucidating the role of CyP-D. This member of the cyclophilin family exhibits peptidyl-prolyl *cis–trans* isomerase (PPIase) activity and is the product of the *Ppif* gene. Notably, CsA binds CyP-D and inhibits its PPIase activity in the same range of concentrations that inhibit the PTP [57,58], yet the isomerase activity is not necessarily involved in pore modulation [59]. The ablation of the *Ppif* gene was obtained in four different laboratories [60–63], and experiments were performed on different models ranging from isolated mitochondria to intact organs. The results provide conclusive evidence that (i) CsA inhibition is mediated by CyP-D and (ii) that CyP-D regulates PTP favouring its opening. Indeed, CsA did not elicit any effect in *Ppif* null mitochondria, while the Ca^{2+} load required to open the PTP increased to match that displayed by wild type mitochondria treated with CsA. However, it must be pointed out that PTP opening was obtained also in *Ppif* null mitochondria, indicating that CyP-D is a relevant factor in PTP modulation but is not required for its formation and opening. The decreased susceptibility to myocardial ischemia displayed by *Ppif*$^{-/-}$*mice* is discussed in Section 3.1.

An alternative model of PTP formation and regulation attributes chaperone-like properties to CyP-D [64]. Accordingly PTP would be formed by clusters of misfolded proteins in a process prevented by CyP-D. Ca^{2+} would perturb the chaperone/cluster complex inducing an open conductance state. This hypothesis might explain the PTP desensitization to Ca^{2+} induced by Hsp25 that could hamper PTP formation by preventing protein misfolding [65], yet it is difficult to see how a highly regulated channel with

defined and reproducible electrophysiological features (including a striking voltage dependence) would be formed by randomly misfolded proteins.

2.4. PTP effectors

Factors affecting the PTP have been thoroughly analyzed by previous reviews [10,26]. Briefly, the most important physiological effectors are as follows:

(i) Divalent cations: The permeability transition is greatly favoured by accumulation of Ca^{2+} ions in the matrix, while it is counteracted by Me^{2+} ions like Mg^{2+}, Sr^{2+} and Mn^{2+} [26,66].
(ii) $\Delta\psi_m$: At physiological membrane potentials the pore favours the closed state, while it can be opened by membrane depolarization [67], although this is not invariably the case. Many effectors are able to modify the threshold voltage. Thus, PTP opening can be obtained by either depolarization, or by changing the threshold potential. On the other hand, mitochondrial depolarization might prevent PTP opening by reducing Ca^{2+} uptake. This concept might hold valid for ischemic tissues.
(iii) Inorganic phosphate favours PTP opening.
(iv) Protons: The probability of PTP opening is sharply increased below and above pH 7.4 [10,26,68]. The inhibitory effect of H^+ is exerted from the matrix side of the inner membrane [66], and is linked to reversible protonation of histidyl residues [69].
(v) Adenine nucleotides: The probability of pore opening is decreased by adenine nucleotides, ADP being more potent than ATP ([70] and references therein).
(vi) ROS: Oxidative stress has long been known to increase the probability of pore opening ([70–74] and reviewed in [25,75,76]). Recent findings indicate that PTP can be targeted by p66Shc-produced peroxides resulting in apoptosis [77,78]. Interestingly, in isolated cardiomyocytes ROS-induced PTP opening was followed by a burst of mitochondrial ROS formation [79].

The latter process of ROS-induced ROS release might be potentially relevant for the amplification of an initial oxidative stress resulting in the recruitment of a great proportion of mitochondria in an injured cell. However, the intracellular spreading of ROS-induced mitochondrial depolarization might be also contributed by the inner membrane anion channel and does not involve the PTP [80]. Therefore, the decrease in $\Delta\psi_m$ induced by oxidative stress [24,81] should not be considered a reliable assessment of the PTP.

Besides these major effectors, numerous compounds have been reported to affect the PTP. Amphipatic cations inhibit PTP opening, whereas the opposite is true of amphipatic anions. Among the latter, a relevant role appears to be played by arachidonic acid. In isolated mitochondria, arachidonic acid acts as a PTP agonist [82], and, in intact cells, causes cell death in response to intracellular calcium overload [83]. We found that the rise in intracellular $[Ca^{2+}]$ does not affect PTP opening per se, but rather activates the cytosolic isoform of phospholipase A_2 prompting a sequence of events whereby the released arachidonic acid causes PTP opening followed by cytochrome *c* release, caspase activation and eventually apoptosis [83].

A new entry in the long list of PTP effectors potentially relevant in the setting of myocardial ischemia is glycine [84], which was shown to prevent PTP opening in isolated mitochondria and intact cells. Therefore, the increased probability of PTP opening that is observed in reperfused hearts could be contributed at least in part by the reduction in glycine content induced by ischemia.

3. PTP and ischemia/reperfusion injury

3.1. Reperfusion injury

At present it is widely accepted that PTP opening contributes to the loss of viability associated with post-ischemic reperfusion. Although PTP opening might be caused by ischemia in the absence of reperfusion [85], the combination of pharmacological and biochemical approaches indicates that PTP opening occurs mostly at the onset of reperfusion. Indeed, conditions associated with post-ischemic reperfusion, such as ROS accumulation, pH normalization and $[Ca^{2+}]$ rise, create an ideal scenario for PTP opening.

Initial support to the role of PTP was provided by pharmacology. In isolated cardiomyocytes, CsA was shown to delay the occurrence of anoxia-induced morphological changes [13] and to prevent the fall in $\Delta\psi_m$ caused by calcium overload [86]. However, the first evidence that CsA protects from reperfusion injury was obtained in perfused hearts by Griffiths and Halestrap [14]. These initial reports were concomitant with and supported by findings obtained in other cell types indicating the crucial role of PTP in cell death [87,88]. Subsequently, the indirect pharmacological evidence was corroborated by direct biochemical approaches. A method was developed based on the mitochondrial uptake of the otherwise impermeant deoxyglucose-6-phosphate (DOG). With this technique, it was possible to demonstrate that PTP opening occurs during reperfusion and its occurrence can be limited by protective interventions [70,89]. Almost concomitantly with the development of the DOG technique, the use of calcein fluorescence was proposed for monitoring PTP opening in intact cells [90]. Besides refining the calcein technique with the introduction of Ca^{2+} quenching [41], we developed another direct approach for the study of PTP in intact hearts based on the efflux and hydrolysis of mitochondrial NAD^+ [44]. The results obtained with this approach confirmed that PTP opening occurs during post-ischemic reperfusion.

Besides representing a useful analytical tool, the loss of mitochondrial NAD^+ has three major consequences that may mechanistically link PTP opening to cell death. Firstly, the reduced availability of this coenzyme inevitably hampers the oxidative metabolism of any substrate, and especially that of fatty acids. The decreased rates of lipid oxidation would increase the availability of CoA acyl esters and carnitine, which affect the function of several transporters [91–93]. Of note, long chain acyl-CoAs [26] are PTP agonists and the accumulation of long chain acylcarnitine has been hypothesized as a mechanism underlying arrhythmogenesis [94]. Secondly, although NAD^+-glycohydrolase only hydrolyzes $NAD(P)^+$, its activity eventually leads to depletion of NAD(P)H thereby decreasing antioxidant defenses. Besides the dependence of GSH content on NADPH, the reduced forms of pyridine coenzymes have been suggested to act directly as scavengers of singlet oxygen [95]. Thirdly, in the cytosol, NAD^+ can be transformed into cyclic nucleotides (such as cADP ribose) that may amplify the initial cell injury by increasing PTP open probability through the release of Ca^{2+} from sarcoplasmic reticulum [96].

More recently, the reduced susceptibility to ischemic injury observed in mice lacking CyP-D provided a definite proof of the role of PTP opening in ensuing irreversible damage not only of the heart [60,62], but also of the brain [63]. The results of this genetic approach lends strong support to the concept that the protection afforded by CsA is related to PTP desensitization. Surprisingly, while reperfusion-induced necrosis was significantly reduced, cells lacking CyP-D did not display a decreased susceptibility to apoptotic stimuli. The conclusion was drawn that PTP opening is involved in necrosis but not in apoptosis [97]. We find this interpretation quite arguable for several reasons. First and foremost, *CyP-D is not the PTP*, but only one of the many factors involved in its regulation, and a permeability transition can occur in its absence [61]. Second, adaptive PTP responses may take place whereby the decreased sensitivity to Ca^{2+} is bypassed by increased sensitivity to oxidative stress [61]. Third, a role of PTP in apoptosis is supported by overwhelming evidence based on cells undergoing apoptosis and on in vivo models of disease [27,31,43,83,98].

3.2. Ischemic preconditioning and postconditioning

Since PTP inhibition prevents reperfusion injury, it was hypothesized that the powerful protection associated with IPC could be attributed to a decreased probability of PTP opening. Following initial indirect evidence [99,100], this hypothesis was validated by using the DOG technique in Halestrap's laboratory [15]. This study also showed that the sensitivity of PTP to Ca^{2+} was higher in mitochondria isolated from IPC-treated hearts than in those from untreated hearts. Therefore, the PTP inhibition observed in situ does likely depend on indirect effects, such as the IPC-induced decrease in calcium overload and oxidative stress [101,102]. These changes (which explain the decreased PTP opening and the consequent maintenance of tissue viability) might result from the stimulation of intracellular signaling pathways [18,81,103–105]. A comprehensive scheme integrating numerous cardioprotective agents has been proposed whereby various pathways converge on glycogen synthase kinase-3β (GSK-3β) resulting in its inactivation. This process would be transduced to mitochondria resulting in PTP inhibition [81]. Convincing evidence was provided to support this hypothesis, yet the mechanisms linking phosphorylation processes occurring in the cytosol with the operation of PTP in the IMM are far from being elucidated. As pointed out by a recent editorial [106], the evidence that GSK-3β or PKCε interact with ANT and VDAC [81,104] is not surprising due to the abundance of these proteins in mitochondria. Furthermore, it is not clear how the phosphorylating activity of cytosolic kinases could reach IMM targets through the OMM. This question also applies to PTP inhibition in mitochondria treated with added PKCε [104]. The explanation that inhibition results from VDAC and ANT phosphorylation is not totally convincing, especially considering that ANT is not required for the formation of a functional PTP [55].

Paradoxically, it has been proposed that PTP opening might be involved in the self-defense mechanisms underlying IPC-induced protection. In fact, CsA and Sanglifehrin A (SfA), another PTP inhibitor that unlikely CsA does not inhibit calcineurin [107], were found to abolish the reduction in infarct size elicited by IPC in perfused rat hearts [24]. Although this finding might disclose novel role(s) for PTP, aspects of this study were questioned by well-grounded criticism [108]. For instance, since both CsA and SfA are likely to remain bound to CyP-D during the ischemic phase, the lack of protection upon reperfusion is surprising. Even more puzzling is the unexpected ability of CsA and SfA to abolish the protection elicited by the uncoupler DNP. Besides these matters of doubt, the available data do not allow elucidating both the role of PTP in IPC protection and the relationships with the other mechanisms proposed so far. In this respect, many reports attribute a central role to the opening of K_{ATP} channels [21,22,109], which have been linked to PTP inhibition by means of $\Delta\psi_m$ decrease and reduced mitochondrial Ca^{2+} uptake [9,110,111]. However, such a relationship cannot explain the result of Hausenloy et al. [24], since PTP inhibition antagonizes the beneficial effects of IPC. On the other hand diazoxide, a K_{ATP} channel opener, increases ROS formation that is required for IPC protection [20,112]. It is tempting to speculate that such an increase in ROS might induce PTP opening, which would then be rapidly resealed by the concomitant drop in pH and rise in [ADP] caused by ischemia. It appears that further studies will be necessary to clarify the possible beneficial consequences of PTP opening that might be useful for protecting the heart.

PTP inhibition has been suggested to be also responsible for the protection afforded by postconditioning, i.e., brief periods of ischemia performed at the onset of reperfusion

[17]. However, the conclusions drawn by this study were found questionable [113]. A central role was attributed to PTP based on the indirect observation that postconditioning and the PTP inhibitor NIM811 confer comparable degrees of protection, as assessed from reduction of infarct size. Importantly, NIM811 was administered just 1 minute before reperfusion and direct evidence of PTP inhibition in situ was not provided. Mitochondria were isolated at the end of the various perfusion protocols showing that the opening of PTP required a higher Ca^{2+} load in mitochondria isolated from protected hearts as compared to those from control hearts. These in vitro experiments do not provide convincing evidence of the role of PTP in postconditioning. In fact, the Ca^{2+} sensitivity was not normalized to the maximum value obtained by adding CsA in all the samples tested, so that comparisons lack an essential internal control. From a more general perspective, it is worth pointing out that any intervention protecting the heart is likely to result in protection of mitochondrial function and structure. Therefore, it is expected that parameters of mitochondrial function are better preserved in mitochondria extracted from protected hearts, including susceptibility to PTP opening. In any case, these measurements do not inform on whether (i) PTP had occurred in situ, and (ii) PTP opening was causally related to myocardial injury. Despite these limits this study confirms that protection can be obtained by treatment with CsA just on reperfusion as originally reported by Hausenloy et al. [100]. The concept that interventions can be used after the onset of ischemia is receiving increasing attention [114] and might be exploited in clinical settings.

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