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Change in lactate production in Myc-transformed cells precedes apoptosis and can be inhibited by *Bcl-2* overexpression

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Abstract As a result of Myc-dependent transcription of the *LDH-A* gene, Myc-transformed cells (Rat1-Myc) exhibit increased lactate production rates (LPR) even under aerobic conditions (the Warburg effect). Recently, the increased susceptibility to stress-induced apoptosis associated with Myc transfection has been linked to the overexpression of the *LDH-A* gene. In this report we demonstrate that the overexpression of the anti-apoptotic protein Bcl−2 in Rat1-Myc cells (Rat1-Myc-Bcl−2) reduces the molar ratio of lactate production to glucose consumption ($Y_{L/G}$). The Bcl−2 induced reduction in $Y_{L/G}$ may be associated with reduced expression of the *LDH-A* gene, or a decrease in LDH-A activity. Stimulation of apoptosis by staurosporine, a protein kinase C inhibitor, reduces the LPR in Rat1-Myc cells in a dose-dependent manner. The staurosporine effect on the LPR is rapid and precedes the execution phase of apoptosis as defined by caspase activation and PARP cleavage. This effect on LPR is completely blocked by Bcl-2 overexpression. Serum starvation alone does not affect the LPR of Rat1-Myc or Rat1-Myc-Bcl-2 cells; however, the effect of staurosporine on the LPR of Rat1-Myc cells is potentiated by serum starvation. These data demonstrate that *Bcl-2* overexpression reduces the $Y_{L/G}$ in Rat1-Myc cells, perhaps via a reduction in the activity or expression of the *LDH-A* gene, and this reduction may desensitize cells to some pro-apoptotic stimuli. The reduction in LPR in response to staurosporine may be an early step in the induction of apoptosis in Rat1-Myc cells. By abolishing the reduction in LPR, Bcl-2 may protect Rat1-Myc cells from staurosporine-induced apoptosis. Moreover, the lack of effect by serum starvation on the LPR supports a model in which serum starvation induces apoptosis through a pathway distinct from that of the staurosporine and glucose-dependent apoptotic pathway(s) in Myc-transformed cells.

Key words: Bcl-2; Apoptosis; Rat1-Myc; Staurosporine; Lactate production

1. Introduction

Alterations in glucose metabolism have been shown to cause apoptosis in Myc-transformed cells by a glucose-dependent apoptotic pathway [1]. It has been hypothesized that this glucose-dependent apoptotic pathway is specifically regulated by c-Myc since apoptosis caused by glucose starvation or exposure to 2-deoxyglucose does not occur in the absence of c-Myc expression [1,2]. Myc transfection has been shown to upregulate expression of the lactate dehydrogenase (*LDH-A*) gene, and ectopic expression of LDH-A is sufficient to sensitize non-Myc-transfected cells to apoptosis induced by glucose starvation or 2-deoxyglucose exposure [1,2]. Bcl-2 overexpression protects Myc-transfected cells from apoptosis induced by glucose starvation, and abolishes the sensitization induced by overexpression of *LDH-A* [1]. The mechanism by which overexpression of *LDH-A* and glucose deprivation induce apoptosis remains to be elucidated. It has been suggested that a reduction in constitutive generation of NAD^+ and lactate by LDH-A and a decrease in regeneration of NADH by the inhibition of glycolysis may be involved [1]. Moreover, changes in NAD^+ and NADH levels may trigger apoptosis by altering the cellular redox state [1]. The mechanism by which Bcl-2 inhibits apoptosis induced by glucose starvation and 2-deoxyglucose exposure is still unknown.

It has been shown that an early event which precedes the proteolytic phase of apoptosis is the release of cytochrome *c* from mitochondria, a process that is blocked by Bcl-2 or Bcl-X_L expression [3–5]. It is, however, unclear whether changes in cellular metabolism (perhaps related to mitochondrial disruption) have a causal effect or are a consequence of apoptosis induction [6]. We reasoned that if the release of cytochrome *c* precedes caspase activation, then metabolic changes related to the disruption of oxidative phosphorylation and perhaps other metabolic pathways might also be observed prior to the onset of cell killing.

In this paper, we describe the effects of staurosporine and/or serum starvation on the LPR of Myc-transformed cells. We also describe the effects of *Bcl-2* overexpression on glucose metabolism as well as the response of Myc-transformed cells to pro-apoptotic stimuli. Our results demonstrate that *Bcl-2* overexpression alters glucose metabolism and reduces the ratio of lactate production to glucose utilization (denoted $Y_{L/G}$) in Rat1-Myc cells under aerobic conditions, that reduction in the LPR is an early event which occurs prior to caspase activation, and that this effect is completely abolished by overexpression of *Bcl-2*. Serum starvation alone does not affect the LPR, but potentiates the effect of staurosporine on the LPR of Rat1-Myc cells.

2. Materials and methods

2.1. Cell lines and culture conditions

Rat1-Myc and Rat1-Myc-Bcl-2 (passage numbers 10–30) cells were grown in DMEM (Gibco) supplemented with glucose (to a final concentration of 20 mM), 10% (v/v) fetal bovine serum (FBS) (Gibco), 100 U/ml penicillin and 100 μg/ml streptomycin (Sigma). The culture

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Abbreviations: FBS, fetal bovine serum; DMEM, Dulbecco's modified Eagle's medium; GCR, glucose consumption rate; LPR, lactate production rate

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medium was replaced daily and cell cultures were split 1:50 every 4 days and replated. On the day of the experiment the medium was aspirated from the flasks and equal volumes of the desired media without or with staurosporine (Sigma) were added to the cells. All the media used in these experiments (including controls) contained 0.1% (v/v) DMSO (added as a vehicle for staurosporine). Samples were removed at various times (depicted in the figures) for glucose and lactate concentration measurements. Glucose and lactate concentrations were measured with standard enzymatic assays performed with an Ektachem DT-60 Analyzer (Eastman Kodak, NY).

2.2. Western analysis for PARP cleavage

On each 60 mm plate, Rat1-Myc cells were washed once with ice-cold PBS and scraped into 1 ml of PBS. Cell pellets were collected by pulse centrifugation and resuspended into 200 μl of ice-cold PBS, followed by the addition of 200 μl of 2×SDS-PAGE gel loading buffer. Cellular DNA was sheared by sonication on ice prior to electrophoresis. Equal amounts of protein lysates were resolved on a 10% SDS-PAGE gel, transferred to nitrocellulose, and probed with anti-PARP antibody (Biomol, 1:1000 dilution) and horseradish peroxidase-conjugated donkey anti-rabbit IgG antibody (Amersham, 1:5000 dilution). Immunostained protein was subsequently visualized via enhanced chemiluminescence (Amersham).

2.3. Statistics

Results are expressed as means ± standard deviations, and are based on multiple independent measurements (n). Statistical significance was determined by Student's t-test.

3. Results

3.1. Myc transfection increases $Y_{L/G}$ in Rat1 cells, and Bcl-2 overexpression reduces the $Y_{L/G}$ in Rat1-Myc cells

Myc transfection has been shown to upregulate the *LDH-A* gene [1], resulting in an increase in LDH-A activity that correlates with an increase in the lactate production to glucose consumption molar ratio ($Y_{L/G}$) under aerobic conditions [2]. In agreement with the observations of Shim and co-workers [2], we observed a shift in the $Y_{L/G}$ of Rat1 fibroblasts which had been transformed by Myc transfection and grown under aerobic conditions (Table 1). Myc transfection increased the $Y_{L/G}$ of Rat1 cells by 50%, from 1.01 ± 0.11 to 1.53 ± 0.10 ($n = 8$). The overexpression of *Bcl-2* in Rat1-Myc cells caused a reduction in $Y_{L/G}$ from 1.53 ± 0.10 to 1.29 ± 0.06 ($n = 8$), a 16% change that was statistically significant ($P < 0.001$). Myc transfection increased the specific LPR and glucose consumption rate (GCR) of Rat1 cells, and *Bcl-2* overexpression reduced both the specific GCR and LPR of Rat1-Myc cells. The GCR and LPR of *Bcl-2* overexpressing cells were, however, significantly higher than those of Rat1 cells (data not shown). The reduction in $Y_{L/G}$ caused by *Bcl-2* overexpression suggests that Bcl-2 may directly alter *LDH-A* gene expression, or reduce LDH-A enzymatic activity. Assuming that an increase in the *LDH-A* expression or LDH-A activity sensitizes cells to pro-apoptotic stimuli [1], it can be argued that a decrease in LDH-A activity introduced by overexpression of *Bcl-2* may desensitize cells and thereby protect them from some pro-apoptotic stimuli. This effect on LDH-A may explain how Bcl-2 protects Myc-transformed cells from glucose-dependent apoptosis.

Table 1
Effects of Myc transfection and *Bcl-2* overexpression on the lactate production to glucose consumption molar ratio ($Y_{L/G}$)

Cell line	$Y_{L/G}$	(n)
Rat1	1.01 ± 0.11	(8)
Rat1-Myc	1.53 ± 0.10*	(8)
Rat1-Myc-Bcl-2	1.29 ± 0.06*,	(8)

Glucose consumption rates (GCR) and LPR were calculated from slopes of the plots of glucose or lactate concentration versus time as shown in Fig. 2. Values represent means ± S.D. based on independent measurements (n) indicated in parentheses.
*Statistically significant difference from the $Y_{L/G}$ of Rat1 cells and statistically significant difference between the $Y_{L/G}$ of Rat1-Myc and Rat1-Myc-Bcl-2 ($P < 0.001$).

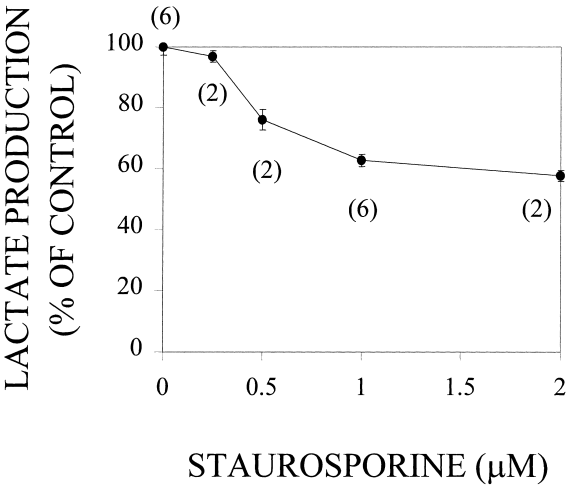

Fig. 1. Effects of staurosporine on the lactate production rate (LPR) of Rat1-Myc cells incubated in serum-free DMEM. Identically seeded duplicate monolayer flasks were exposed for 8 h to serum-free DMEM containing 0.00, 0.25, 0.50, 1.00 and 2.00 μM staurosporine. For controls (no staurosporine), and cells exposed to 1 μM staurosporine, four additional flasks were used, bringing the number of independent measurements to $n = 6$ (n is indicated in parentheses for each point). Media samples were removed from each flask at time zero and approximately every 2 h thereafter for lactate concentration measurements. LPR was calculated from the slope of the plot of lactate concentration versus time as shown in Fig. 2. For each point, error bars represent the standard deviation based on the number of independent measurements (n) indicated in parentheses. Where not seen, error bars are within the marker.

3.2. Effects of serum starvation and/or staurosporine on LPR in Rat1-Myc and Rat1-Myc-Bcl-2 cells

Exposure of Rat1-Myc cells to staurosporine reduced LPR in a dose-dependent manner (Fig. 1). For the data presented in Fig. 1, the LPR was calculated based on the increase in lactate concentration with time over a period of 8 h. The increase in lactate with time was highly linear over the 0–8 h time interval for both the control and staurosporine-treated cells. The slope of the straight line in staurosporine-treated cells was always lower compared to control cells. The above observations suggest that the effect of staurosporine on LPR is rapid, and that shortly after exposure, staurosporine-treated cells produce lactate at a constant and lower rate than the control cells. It is important to note that in our experimental system the reduction in LPR must occur well before a reduction in the lactate concentration in the incubation media is observed. In the data shown in Fig. 2c, the earliest time point for which a statistically significant difference in lactate concentration was observed in staurosporine-treated versus control cells was after 3 h of exposure. The earliest time point for which such a difference was observed throughout our experiments was after 30 min of exposure.

Serum starvation had no effect on LPR of Rat1-Myc cells as calculated from the slopes of the curves (Fig. 2a). Similar

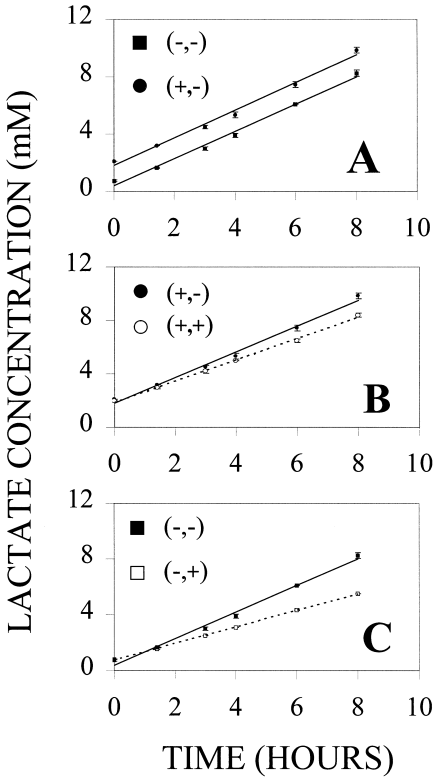

Fig. 2. Lactate production of Rat1-Myc monolayers incubated in serum-free, and serum containing (10% v/v) DMEM media in the presence (1 μM) or absence of staurosporine. Lactate concentration profiles in flasks incubated in: (a) serum-free (−,−) and serum-containing (+,−) DMEM in the absence of staurosporine; (b) serum-containing DMEM in the absence (+,−) or presence (+,+) of 1 μM staurosporine; (c) serum-free DMEM in the absence (−,−), or presence (−,+) of 1 μM staurosporine. Error bars represent the standard deviation from the mean measurement performed on identically seeded duplicate flasks ($n=2$). Where not seen, error bars are within the marker. The slope of the lines reflects the lactate production rate (per flask). The higher lactate concentration at time zero in cultures incubated in serum-containing media reflects the presence of lactate in serum.

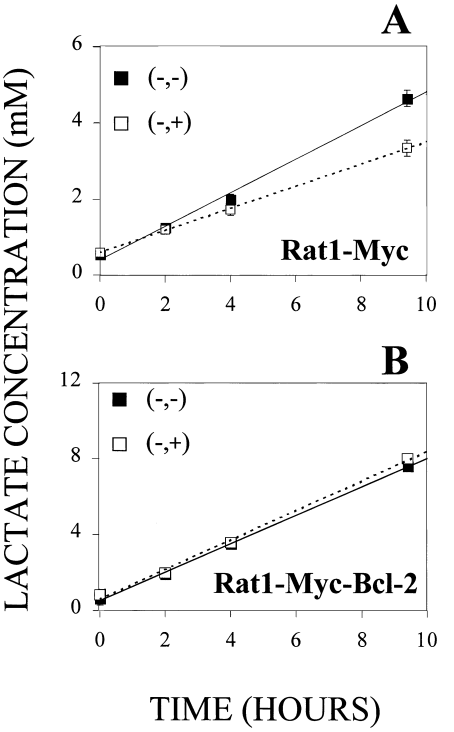

Fig. 3. Effect of staurosporine on lactate production in Rat1-Myc and Rat1-Myc-Bcl-2 monolayers incubated in serum-free media. Time-dependent lactate production in Rat1-Myc (a) and Rat1-Myc-Bcl-2 (b) monolayers incubated in serum-free DMEM in the absence (−,−), or presence (−,+) of 1 μM staurosporine. Error bars represent the standard deviation from the mean measurement performed on identically seeded quadruplicate flasks ($n=4$). Where not seen, error bars are within the marker. The lactate production rate is calculated from the slope of the line.

results were obtained for Rat1-Myc-Bcl-2 cells (data not shown). A staurosporine-mediated reduction in LPR was observed in cultures incubated in serum-supplemented media (Fig. 2b), and this effect was further potentiated in serum-free media (Fig. 2c, Table 2). It is not clear from these experiments whether serum starvation potentiated the effect of staurosporine on the LPR synergistically, or whether serum attenuated the effect by reducing the bioavailability of staurosporine through serum protein binding.

Table 2
Staurosporine effects on lactate production of Rat1-Myc cells incubated in serum-free or serum containing media

Serum	Staurosporine	Lactate production rate	
		% of control	(*n*)
+	−	100.0 ± 4.1	(6)
−	−	98.4 ± 3.1	(6)
+	1 μM	83.4 ± 1.8*	(6)
−	1 μM	61.7 ± 2.1*,	(6)

Values represent means ± S.D. based on the number of independent measurements (*n*) indicated in parentheses.
*Statistically significant difference from the control, statistically significant difference between the rates measured from flasks containing cells incubated in 1 μM staurosporine in either the absence or presence of serum ($P<0.001$).

Differences in the effect of staurosporine on LPR of Rat1-Myc (sensitive) and Rat1-Myc-Bcl-2 (resistant) cells are presented in Fig. 3a,b. There was no reduction in the LPR in Rat1-Myc-Bcl-2 cells incubated in the presence of 1 μM staurosporine, in contrast to the substantial reduction observed in Rat1-Myc cells (Figs. 1 and 3b, Table 2).

Exposure of both Rat1-Myc and Rat1-Myc-Bcl-2 cells to staurosporine caused a significant change in cellular morphology (rounding-up) which was not observed in untreated cells. No cell detachment was observed after a 4 h treatment with staurosporine. The change in morphology is unrelated to the effect on the LPR since it was also observed in cells overexpressing Bcl-2 (data not shown).

3.3. *Staurosporine-mediated decrease in lactate production precedes onset of PARP cleavage*

At the onset of apoptosis, caspases become activated by an autocatalytic event coupled to cytochrome *c* release from the mitochondria and formation of a complex with APAF-1 [7,8]. Activated caspases play a critical role in ensuring apoptosis-associated morphological changes [9–12]. Cleavage of PARP is absolutely dependent on caspase activation, and is a hallmark of apoptosis [13]. While many metabolic changes are associated with apoptosis [6], it is still unclear whether these changes are the outcome of induction of apoptosis (especially alterations in mitochondrial function), or whether they serve

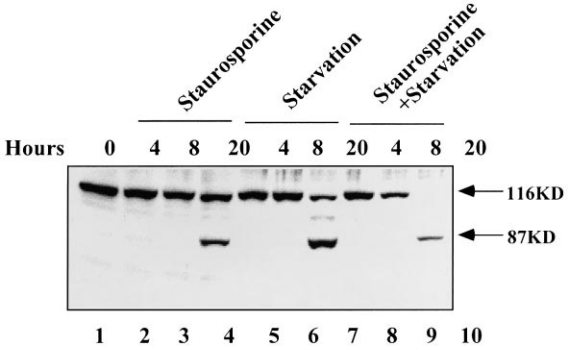

Fig. 4. PARP cleavage in staurosporine-treated or serum-starved Rat1-Myc Cells. Subconfluent Rat1-Myc cells were treated with staurosporine (1.0 μM), serum starvation (0.1% DMEM) or the combination of staurosporine plus serum starvation. Cells were collected and lysed at different periods of time (0–20 h). Cell lysates were separated on 10% SDS-PAGE gel, and Western blot was performed as described in Section 2. The arrows indicated the 116 kDa PARP precursor and 87 kDa cleavage product.

as signals that promote apoptosis [14–16]. To determine the order of events at the onset of apoptosis, the cleavage of PARP was examined in Rat1-Myc cells at different time periods following initiation by various apoptotic stimuli. In both staurosporine-treated and serum-starved cells, PARP cleavage was observed at 20 h but not at 8 h following stimulation (Fig. 4). Furthermore, in a series of experiments with Rat1-Myc cells, the release of cytochrome *c* was not observed at 4 h, but was detectable after 8 h exposure to 1 μM staurosporine (data not shown). The combination of staurosporine and serum starvation induced both PARP cleavage and degradation (Fig. 4, lane 10). Nevertheless, PARP cleavage still occurred at a late time point and not prior to 8 h following stimulation. No PARP cleavage was observed in staurosporine treated Rat1-Myc-Bcl-2 cells, even after 20 h of exposure (data not shown). Since the effect of staurosporine on LPR is rapid (i.e. it must have been initiated prior to 30 min of exposure, see previous discussion), precedes cytochrome *c* release and the onset of PARP cleavage, the reduction of lactate production by staurosporine could not be a consequence of apoptosis. Furthermore, the staurosporine-mediated decrease in lactate production was completely blocked in Rat1-Myc cells overexpressing the anti-apoptotic gene *Bcl-2* (Table 3) suggesting that the metabolic pathway for lactate production might be involved in early signaling events that would precede the onset of apoptosis.

4. Discussion

It has been demonstrated that Myc transfection upregulates the *LDH-A* gene, LDH-A activity, and increases the lactate production to glucose consumption molar ratio ($Y_{L/G}$) in Rat1 cells [1,2]. Myc-transformation has been associated with an increased susceptibility to stress-induced apoptosis, which has been recently linked to the overexpression of the *LDH-A* gene [1]. Overexpression of the anti-apoptotic gene *Bcl-2* has been shown to protect Myc-transformed cells from stress-induced apoptosis [1,2]. The mechanism by which Bcl-2 protects cells from apoptosis is largely unknown and the subject of intense investigation. Our observation that overexpression of the anti-apoptotic protein Bcl-2 in Rat1-Myc cells reduces the $Y_{L/G}$ is consistent with a reduction in the expression of the *LDH-A* gene or a reduction in LDH-A activity. Based on a recent report demonstrating that an increase in *LDH-A* expression or LDH-A activity sensitizes cells to pro-apoptotic stimuli [1], it can be argued that a reduction in *LDH-A* expression and/or LDH-A activity may 'desensitize' Rat1-Myc cells to such stimuli. Such a reduction caused by overexpression of *Bcl-2*, may partly explain its ability to protect Myc-transformed cells from glucose-dependent apoptosis. It is not clear from our results whether the entire population of cells that are exposed to staurosporine reduce their LPR by 40%, or whether cells committed to apoptosis (40% of total cells) completely stop lactate production. Either scenario would generate the same effect on LPR as observed here. It is worth noting that in a study with a different cell line (HT-29 human colon adenocarcinoma), approximately 40% of cells underwent apoptosis upon exposure to 1 μM staurosporine for 48 h [17].

Our results demonstrate a clear effect of staurosporine on the LPR of Rat1-Myc cells. The effect of staurosporine on LPR was rapid and preceded the onset of the execution phase of apoptosis as established by measuring PARP cleavage. The above observations are consistent with the suggestion that metabolic changes may be early signaling events, and may be responsible for generating a signaling cascade which triggers the execution phase of apoptosis. This does not imply that other metabolic changes cannot be the consequence of apoptosis after the onset of PARP cleavage.

One may hypothesize that the LPR may be an important determinant in the signaling of staurosporine-induced apoptosis. This hypothesis is consistent with a model in which changes in glycolytic flux through LDH-A that result in staurosporine-induced apoptosis may be identical or share common signaling intermediates with the recently described glucose-dependent apoptotic pathway [1]. The fact that overexpression of *Bcl-2* blocks both the reduction in LPR and apoptosis, suggests that the anti-apoptotic effect of Bcl-2 may be mediated via modulation of the LPR. It is unlikely that differences in the effect of staurosporine are related to the

Table 3
Effect of staurosporine on lactate production in Rat1-Myc and Rat1-Myc-Bcl-2 monolayers incubated in serum-free media

Cell line	Staurosporine	Lactate production rate	
		% of control	(*n*)
Rat1-Myc	−	100.0 ± 2.1	(4)
	+	64.5 ± 1.7*	(4)
Rat1-Myc-Bcl-2	−	100.0 ± 0.2	(4)
	+	104.2 ± 5.7^{NS}	(4)

Conditions: monolayers incubated in serum-free DMEM in the absence (−) or presence (+) of 1 μM staurosporine. Values represent means ± S.D. based on the number of independent measurements (*n*) indicated in parentheses.
*Statistically significant difference from the control ($P < 0.001$); NS = not significant.

multiple drug resistance (MDR) phenotype since both Rat1-Myc and Rat1-Myc-Bcl-2 cell lines express equal levels of P-glycoprotein (data not shown). Our data also suggest that although overexpression of *Bcl-2* blocks staurosporine-mediated reduction in LPR, it does not block other metabolic effects (manuscript in preparation).

Serum starvation alone had no effect on the LPR of Rat1-Myc cells despite the fact that it could induce apoptosis. This observation is consistent with the hypothesis that serum starvation induces apoptosis via a mechanism that is independent of the glucose-dependent pathway [1]. The effect of staurosporine on the LPR, however, was potentiated by the absence of serum from the incubation media. Further work is required to establish whether serum starvation potentiates the effect of staurosporine synergistically or whether serum reduces the bioavailability of staurosporine via protein binding.

In summary, the results presented in this paper demonstrate that staurosporine affects the LPR of Rat1-Myc cells prior to the onset of apoptosis, and that this effect is abolished by the overexpression of *Bcl-2*. In addition, *Bcl-2* overexpression reduces $Y_{L/G}$ in Myc-transfected cells suggesting that a reduction in the activity or expression of *LDH-A* occurs in these cells. These results are compatible with an involvement of LDH-A in the mechanism of staurosporine-induced apoptosis, and that staurosporine may share a common signaling pathway with the glucose-dependent apoptosis pathway induced by Myc. The data suggest that the protective effect of Bcl-2 may be mediated via desensitization of the glucose-dependent pathway either by a reduction in the activity or expression of *LDH-A* in the cells, and/or by abolishing the effect of staurosporine on the LPR, which in turn may enable cells to maintain their redox state.

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