Jurnal
Kardiologi Indonesia
J Kardiol Ind 2007; 28:404-410
ISSN 0126/3773

Basic Research

Dynamic Interaction Between Organellas In the Management of Cytosolic Calcium Huvecs Exposed to 22 mM Glucose With Different Period Exposure

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Background. In our previous research, when cell culture were exposed to high glucose, this will cause the increase of H2O2. At the exposure to 22 mM glucose on 3rd day, the increase of H2O2 that induced the activation of Phospholipase C (PLC) have caused 1P3 (Inositol tri-phosphate) mobilizing the release of Ca^{2+} from the depo Endoplasmic reticulum (ER). Thus, causing the increase of cytosolic Ca^{2+}. Giving thapsigargin (TG) will cause significant increase in Cytosolic Ca^{2+} so that the most contribution to the increasing of Cytosolic Ca^{2+} derives from the ER . On the 7th day exposure, H2O2 played the same role as TG, causing direct incease in Cytosolic Ca^{2+} and an addition of Ca^{2+} free/buffer ethyleneglyco bis (ßaminoethyl ether).& NNN'N'– tetraacetic acid (EGTA) caused significant decrease of cytosolic Ca^{2+} basal and the greatest contribution to the increase of cytosolic Ca^{2+} on the 7th day, comes from extracellular. Administrating Cyclosporin A (CSA) 10 µM on the 9th day, caused significant decreasing on cytosolic Ca^{2+} basal, the ability of CSA in decreasing Ca^{2+} basal concentration was less than the 3rd and 7th days. At a high glucose condition with different length of exposure, a change of new cytosolic Ca^{2+} homeostatic regulation occurred and this enable a change in the dynamic interaction among ER, extracellular and mitochondria.
Method. HUVECs culture exposed to 22 mM glucose for 3, 7 and 9 days. The cells were incubated with FURA2-AM. The evaluation of fluorescence cytosolic Ca^{2+} was done by epifluorescence Nikon digital camera-computerized analyser. To measure the cytosolic Ca^{2+} concentration we use Histogram Image Corel Draw Photo Paint 12.
Result. Exposure to glucose 22mM on the 3rd day (65.4 ± 12.2) it showed the increase of cytosolic Ca^{2+} by giving Ca^{2+} free/EGTA 1 mM and CSA 10 mM caused the decrease of cytosolic Ca^{2+} (33.2 ± 4.47) TG1µM and CSA caused the decrease of cytosolic Ca^{2+} basal (53.07 ± 2.75) and Ca^{2+} -free/EGTA, TG and CSA (68.59 ± 5.71). On the 7th day exposure (92.74 ± 7.66) the decrease of cyto -solic Ca^{2+} basal occurred at the giving of Ca^{2+} -free/EGTA, TG (50.52 ± 9.23). EGTA and CSA (45.59 ±6.2). TG and CSA (73.55 ± 7.30), Ca^{2+} -free/EGTA and TG much more decrease the concentrate of cytosolic Ca^{2+} basal (17.58 ±4.5). On the 9th day of exposure to glucose (72.32 ±7.46), the giving of Ca^{2+} -free/EGTA, TG and CSA(35.76 ± 5.25) have caused the decrease of cytosolic Ca^{2+} basal.
Conclusion. HUVECs culture exposed to 22mM glucose will cause the increase in H2O2and cytosolic Ca^{2+} basal. ER, mitochondria and extracellular regulate the Cytosolic Ca^{2+} and a dynamic interaction occurred among them to obtain a new homeostatic. (J Kardiol Ind 2007;28:404-410)

Keywords: high glucose, H2O2, cytosolic Ca^{2+}, endoplasmic reticulum, mitochondria

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At physiological homeostatic condition cytosolic Ca^{2+} was regulated by the plasma membrane, Endoplasmic Reticulum (ER), mitochondria and non membrane

protein. Such regulation occurred through three main mechanism namely intracellular increase in Ca^{2+}, intracellular decrease, and buffering Ca^{2+},(Moorena *and* Kinneb, 1998, Parekh, 2003. Many agonized receptor such as bradychinine, histamine, trombine and adenocine triphosphate (ATP) have been proven to be the cause of the raise of biphasic cytosolic Ca^{2+} (Kwan *et al*, 2003; Kim *et al*, 2004). Early raise of cytosolic Ca^{2+}, which temporary illustrates the release of ion Ca^{2+} from the depo(ER) which is induced by inocytol 1,4,5-trisulphate (IP3) (Marshal *and* Taylor, 1992; Swatton *and* Taylor, 2002; Parekh, 2003) followed by the influx of Ca^{2+} from extracellular through the activated canal calcium release activated current (CRAC) (Berridge, 1998; Putney, 2001) with a very selective flow against Ca^{2+} (ICRAC) (Hoth *and* Panner, 1997).

Many research have been conducted to show the dynamic interaction between ER, Extracellular and Mitochondria as the main regulator at homeostatic intracellular Ca^{2+}. Parekh (2002) show that the way of taking the Ca^{2+} by mitochondria may reduce the work of IP3 to stimulate the influx of Ca^{2+} from extracell. Hajnóczky *et al*., (2000), There are local communication influx of Ca^{2+} through cell membrane, ER and mitochondria to take Ca^{2+}, so that a further research on dynamic interaction between ER, extracellular and mitochondria at human umbilical vein cells (HUVECs) with high glucose exposure is necessary.

Permatasari (2003) reported that the endothelial cells (HUVECs) culture exposed to 22mM glucose during 3,5,7,9 days exposures will raise the cytosolic Ca^{2+} concentration caused by the raise of H2O2.

At the First stage research, the result of measuring the content of H2O2 in HUVECs culture exposed by 22mM glucose by using colorimetric hydrogen peroxide, shows a significant raise at the exposure on the 3rd , 7^{th} and 9th days, the cytosolic Ca^{2+} shows an increase too. There was no sifnificant difference with the 5 mM glucose given ATP 10 µM, this proofs that H2O2 as an intracellular messenger at endothelial cell is the same as ATP. It can stimulate the release of Ca^{2+} from the ER depo which is sensitive to IP3 By adding Thapsigargin (TG) as a specific SERCA blocker1 µM will cause the raise the cytosolic Ca^{2+} basal very significantly compared to the exposure of high glucose on the 7th and9th days so, it can be concluded that the main contribution to the raise of cytosolic Ca^{2+} endothelial cell at the exposure to glucose on the 3rd day derived from ER depo.

At the Second stage Research, the administration of Ethyleneglycol bis (ß- aminoethyl ether) N,N.N, - tetraacetic acid (EGTA) 1 mM as Ca^{2+} chelator at media Ca^{2+} free condition HUVECs exposed to high glucose, on the 7th day significantly decrease the concentrate of cytosolic Ca^{2+} basal when compared to the exposure of glucose on the 3rd and 9th days, so it can be concluded that the main contribution to the raise of cytosolic Ca^{2+} on the 7th day derives from the extracellular.

On the exposure of glucose on the 9th day, a significant decrease occurred from the cytosolic Ca^{2+} concentration when compared to exposure of on the 7^{th} day. This condition is predicted that there is a new change in the homeostatic regulation, where mitochondria as the main regulator in decreasing the cytosolic Ca^{2+} concentration, so that in the Third stage research, by adding Cyclosporin A (CSA) 10 µM as blocker to MPT pore, on the 9th day there was a significant decrease in the cytosolic Ca^{2+} basal. At a high glucose condition with different length of exposure, a new change in homeostatic cytosolic Ca^{2+} regulation occurred, which enables a dynamic interaction change amongs ER, Extracellular and mitochondria.

Thus, in the fourth stage research, a combination is used by administering buffer Ca^{2+} free/EGTA, CSA and TG just to know the role of each treatment to the organel such as ER, Extracellular and mitochondria endothelial cells exposed to a high glucose on the 3r, 7^{th} and 9th days.

Methods

Experimental research is conducted by using endothelial (HUVECs) culture exposed to 22mM dosage of Glucose for 3, 7 and 9 days. For combination, of Ca^{2+} free/EGTA 1mM 30 minutes incubated CSA 10 µM &TG 1 µM for 15 minutes, then each is treatment is evaluated with profile cytosolic Ca^{2} profiles with FURA2-AM. Measuring Cytosolic Ca^{2+} : Done under a fluorescence Nikon Optiphot-2 microscope, UV-2A filter connected to Nikon Digital Camera-computerized analyzer at 340 nm and 380 nm wave length of excitation. Calculation of the content of cytosolic Ca^{2+} by using Histogram Image Corel Draw Photo-Paint-12.

Data Analysis: The research is conducted factorially using complete random sampling one way classification ANOVA. the Tuckey's test. Descriptive analysis

Results

At a condition where the exposure of 22mM glucose occurred on the 3rd day (**Figure 1A**) shows the content of cytosolic Ca^{2+} basal (65.4+12.21).

When HUVECs is incubated with Ca^{2+} Free/ EGTA combined with TG, this will cause a raise in the cytosolic Ca^{2+} basal (82.19+7.03), p‹ 0.001. This result shows the role of TG in increasing cytosolic Ca^{2+} basal . To know the role of ER and mitochondria a combination of Ca^{2+}-Free/EGTA and CSA is administered, which will result in the decreasing of cytosolic Ca^{2+} basal (33,2±4,47), p‹ 0.001.This proofs that the administration of CSA will decrease the cytosolic Ca^{2+} concentration. From the research, it is found that given TG and CSA will cause an extreme decrease in cytosolic Ca^{2+} basal (53.07+2.75) with Δ 39.24 p‹ 0.001, compared to combination of EGTA and CSA with Δ decrease for 25.08, and of Ca^{2+} - Free/EGTA, TG and CSA (68.59+5,71), with ?decrease for 14,60.

At the exposure of 22 mM glucose on the **7th day**, to know the role of the influx of Ca^{2+} from the extracelullar, a combination of Ca^{2+} Free/EGTA and TG (**Figure 1B**), (50.52+9.23) that shows significantly decrease p‹ 0.001.

While a combination of EGTA and CSA without TG (45.59±6.2) will decrease the cytosolic Ca^{2+} basal, with Δ. 14,60 in compared to TG and CSA (73.55+7.30) with Δ decrease of 23.30. When CSA is added at EGTA, and TG will even more ↓ the cytosolic Ca^{2+} basal concentration (17.58+4.54) with Δ 33.04. This shows that the effect of an accumulation of EGTA and CSA in decreasing the cytosolic Ca^{2+} needs TG as a blocker to the SERCA pump.

On the **9th day**, (**Figure 1 C**), the giving of Ca^{2+} Free/EGTA and TG will decrease the cytosolic Ca^{2+} basal concentration (43,54±5,39) , p‹ 0.001. At the administration of EGTA and CSA (48,58+3,44) shows the raise of cytosolic Ca^{2+} when compared to EGTA and TG with Δ raise of 3.11, the administration of TG and CSA (52.1+5.8) will cause a decrease in cytosolic Ca^{2+} with Δ. 23,49 ,and administration of Ca^{2+} - Free/EGTA , TG and CSA (35.76+5.25) will cause a decrease of cytosolic Ca^{2+} with Δ 7,78 . From many combination of administration on the 9th day it can be concluded that the power of CSA in decreasing cytosolic Ca^{2+} requires the role of TG as a SERCA pump blocker.

From the three results mentioned above, the difference of factors that affect the cytosolic Ca^{2+} basal can be seen from day to day On the 3rd day, the simultaneous administration of TG, EGTA and CSA in maintaining the cytosolic Ca^{2+} concentration basal if TG is not given on the exposure of EGTA and CSA, will decrease of the cytosolic Ca^{2+} basal. While on the 7th and 9th days the effect of adding TG to the exposure of HUVECs with EGTA and CSA will precisely decrease the cytosolic Ca^{2+} basal. This result shows the difference of TG working effect on the 3rd day when compared to on the 7th and 9th days. The combination in giving Ca^{2+} free/EGTA and CSA significant decrease of fluoresence cytosolic Ca^{2+} is seen because of the CSA's influecence and starting to raise at the 7th day to 9th day exposure , it is assumed that there is a decrease in the power of CSA to decrease the cytosolic Ca^{2+} at this condition.

The administration of a combined TG and CSA on the 3rd, 7th and 9th days will decrease the fluorescence cytosolic Ca^{2+} compared to the exposure of 22mM glucose only, this shows that the role of TG as a blocker to SERCA consistently increase the power of CSA to intake Ca^{2+} by mitochondria. This condition is also seen at the exposure of 22mM glucose with an administration of Ca^{2+} - free/EGTA and TG and CSA combination.

The administration of a combined TG and CSA on the 3rd, 7th and 9th days will decrease the fluorescence cytosolic Ca^{2+} compared to the exposure of 22mM glucose only, this shows that the role of TG as a blocker to SERCA consistently increase the power of CSA to intake Ca^{2+} by mitochondria. This condition is also seen at the exposure of 22mM glucose with an administration of Ca^{2+} - free/EGTA and TG and CSA combination.

Discussion

Typically the endothelial cells did not express the Ca^{2+} voltage gated Channel at cells membrane but rely on the alternative channel Ca^{2+} influx to cytosol to maintain the homeostatic Ca^{2+}. Hormone and neurotransmitter trigger the phosphoinositol channel, resulting in the increase of byphasic intracellular Ca^{2+} concentration; at the beginning there was an instant release of Ca^{2+} from the ER depo then further followed by a plateau phase that is Ca^{2+} influx from extracellular which was needed to arrange all cellular process which depends on Ca^{2+} such as exocitosis process, the growth

and proliferation of cells. Hoth *and* Panner (1992) show a flow of Ca^{2+} entering into the mast cell culture having close relation with the release of Ca^{2+} from the ER depo, called Ca^{2+} *release activated* Ca^{2+} *current* (I*CRAC).* I*CRAC* is a non voltage gated, inwardly rectifying, very selective for Ca^{2+} with very positive reversal potential (> + 60mV) Parekh *and* Panner, 1997). I*CRAC* can go through into a channel named calcium-released activated channel (CRAC).

From the result of the II stage research, it is found that the administration of Ca^{2+} - free/EGTA glucose for 3 days significantly decrease the cytosolic Ca^{2+} basal..Adding TG will cause a significant increase in cytosolic Ca^{2+} basal (**Figure 2.1A**). TG mobilized Ca^{2+} from the depo to cytosol without being affected by the increase of IP3. The same profile was also reported by Hu et al. (1998) on human aortic endothelial cells culture stimulated with H2O2 100 µM and incubated with Ca^{2+} - free/EGTA 1 mM for 30 minutes cause non significant decrease of cytosolic Ca^{2+} basal. Addition of TG1 µM may stimulate a huge Ca^{2+} release from ER depo to cytosol, that cause the increase of cytosolic Ca^{2+} concentration. From this research it is seen that the main contribution to the increase of cytosolic Ca^{2+} basal derives from the ER depo triggered by IP3 as a result of the accumulation of H2O2 because of the exposure of high glucose and further activate Ca^{2+} influx from extracellular. It is conform to **Figure 3**, morphologically on the 3rd day, ER plays an important role in ion Ca^{2+} regulation. The release of Ca^{2+} from the depo and the blocking of SERCA attributable to the activation of I*CRAC* by TG so that an increase of Ca^{2+} influx from extracellular occurred, which will further cause an accumulation of cytosolic Ca^{2+} (Zweifach *and* Lewis, 1995; Hoth *and* Panner, 1992; Parekh 2001; Parekh, 2002). Parekh (2002) has the opinion that the increase of the mobility speed of Ca^{2+} from cytosol by other organella could become an effective mechanism as it can compete in an effective way with SERCA pump, thus will decrease the speed and the refilling degree into the ER depo.

Mitochondria becomes the main focus based on 3 reasons; (I) from various proofs already recognized, under a physiologic condition mitochondria can significantly take Ca^{2+} from cytosol when increase through a relatively low afinity from uniporter Ca^{2+} , in picture 3 that mitochondria regulates cytosolic Ca^{2+} with Ca^{2+} influx mechanism from cytosol and Ca^{2+} efflux from mitochondria (picture 3 with white arrow mark) (ii) physically the position of mitochondria is close to ER and there occurred interaction between the organelles in handling Ca^{2+}. Many researches reported that there was a coordination between Ca^{2+} signal from ER and mitochondria, facilitated through its proximity mitochondria to the place of releasing Ca^{2+} by ER .For such purpose in this research a combination of EGTA and CSA at HUVECs culture exposed to 22 mM glucose for 3 days (Picture 1A, D) is administered, decreases the cytosolic Ca^{2+} basal significatly when compared to Ca^{2+} -free/EGTA self.

The HUVECs Cytosolic Ca^{2+} with FURA2-AM Staining

Picture 4 proofs that endothelial cells exposed to 22 mM Glucose on the 3rd day by using FURA2-Am and mitoracker Red staining, shows a clear view of the increased Ca^{2+} in the cytosolic microdomain (yellow arrow) and it is assumed to be a strategic location of Ca^{2+} influx from plasma membrane, a location to release Ca^{2+} from ER depo and mitochondria. (iii) Mitochondria can intake Ca^{2+} through the Ca^{2+} influx channel over the plasma membrane. Mitochondria and ER compete in increasing the Ca^{2+} concentration in cytosol and these are the 2 important organellas in handling Ca^{2+}.

Smaili *et al*.;(2001) reported that CSA is incapable in stimulating the intake of Ca^{2+} from cytosol by mitochondria because the cytosolic Ca^{2+} concentration is under the basal concentration. (Csordas *et al*.; 1999; Hajnoczky *et al*; 2000, Smaili *et al,* 2001; Brookes *et al*, 2004).

For further research a combination administration of TG and CSA . significant decrease of cytosolic Ca^{2+} basal occurred by Δ 39,2. On the contrary, a combination of Ca^{2+} -free/EGTA, TG and CSA, in endothelial cells exposed to high glucose for 3 days, a cytosolic Ca^{2+} decrease occurred for Δ14.60. It is assumed that: (i) An increase of cytosolic Ca^{2+} attributable to the activity of TG to ER, blocking the SERCA pump, TGs enables CSA to decrease cytosolic Ca^{2+} basal. By using fluorescence tetra methyl-rodhamine ethylesther (TM REE) indicator to measure the potential membrane ($\Delta\Psi m$), The raise of $\Delta\Psi m$ induced by CSA is a parameter mediator to the high speed in the taking of Ca^{2} by mitochondria (Kowaltowski *et al*; 2000, Brustovesky *and* Dubinsky, 2000; Smaili *et al*, 2001). (ii) The existence of a maximal extracell Ca^{2+} influx will increase the Ca^{2+} in cytosolic microdomain which is close to the plasma membrane, will speed the intake of Ca^{2+} by mitochondria located in this area. Sharma et al, 2000 showed that 90% units

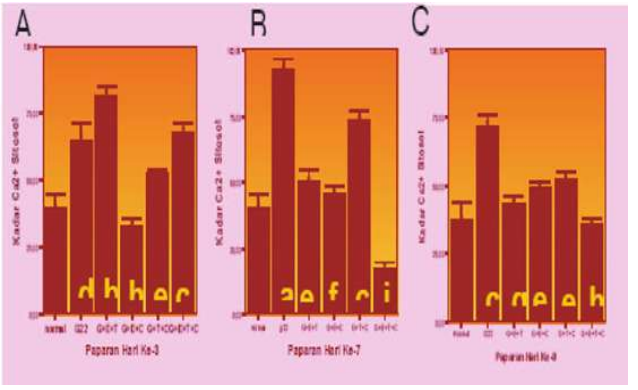


Figure 1. The profilesn ofCytosolicl Ca2+ basal at Huvecs culture exposed to 22 mM glucose after given the combination of TG, EGTA/ Free Ca2+ and CSA at the length exposure on 3rd (A) ,7th (B)and, 9th days(C) Bar showing mean. Error bar showing mean ± 0.5 SD,* p<0.001, n=20. Mean followed by the same letter means d no significant difference with p=0,05

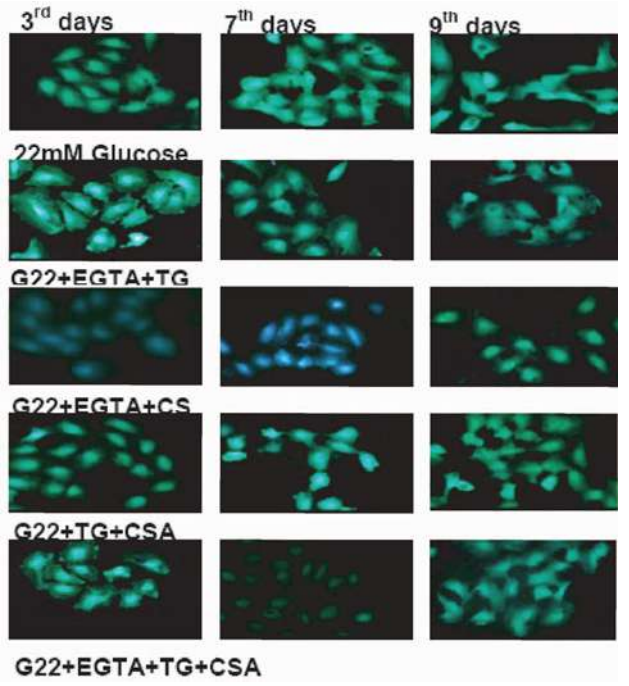


Figure 2. The fluorescence profile of cytosol Ca^{2}+ at various treatments. The change of fluorescence clearly shown at a 22 mM/L glucose treatment with each length of exposure. An increase of fluorescence cytosolic Ca^{2}+ only at 22 mM glucose exposure starting on the 3rd day to 7th day and starting decreasing on the 9th days.

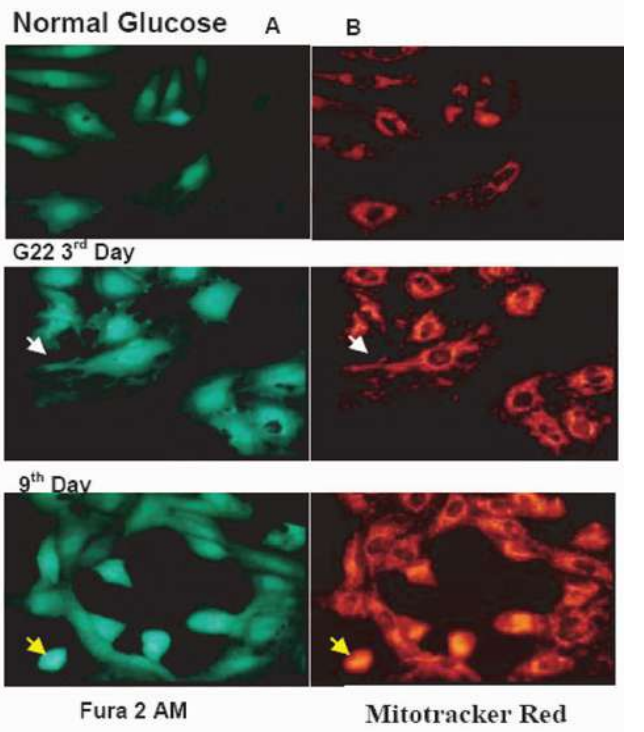
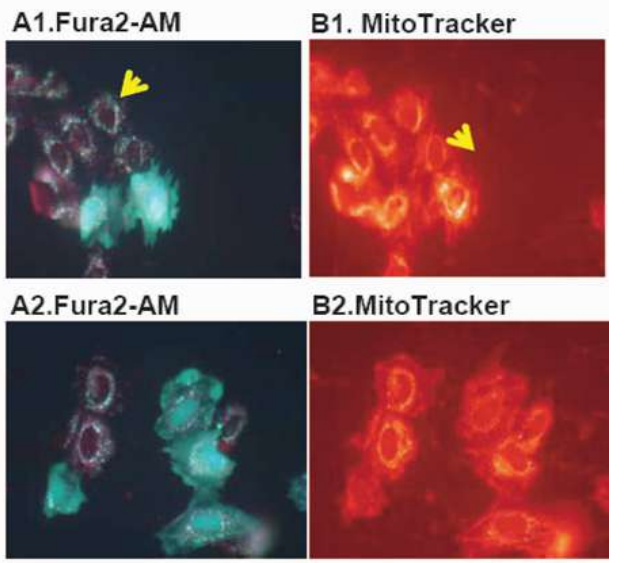


Figure 3. The profile of cytosolic with double staining FURA2-AM and mitoTracker Red under a fluorescence Nikon optophot-2 microscope on the exposure of glucose on the 3rd , 7th, and 9th days.
In Picture A, green represents the distribution of cytosolic Ca^{2}+ with 340 nm length ware excitement and 380 nm. B. Red illustrates the mitochondria by using mitoTracker red (with 550 nm excitement wave.



Picture 4. FURA2-AM and Mitotracker staining, exposesure of 22mM Glucose on the 3rd day.

of Ca^{2+} released from (ER depo) are closely located to mitochondria. (iii) Picture 1B, D and 2 showed that at 22mM glucosed HUVECs culture condition for 7 days, the Ca^{2+} influx channel from extracell was examined using Ca^{2+} -free/EGTA & TG,shows significantly decrease the cytosolic Ca^{2+} basal concentration. The main contribution to the increase of cytosolic Ca^{2+} from extracells. TG is not sufficiently capable in increasing the cytosolic Ca^{2+} basal. On 7th day, H2O2 established from being highly glucosed function as TG, directly emptying the Ca^{2+} ER depo, without affecting IP3 and immediately activate Ca^{2+} influx from extracellular (Hu *et al*., 1998). It is assumed that as a direct effect from oxidant to the binding site of Ca^{2+} enzyme – AT Pase (Xu *et al*., 1997). H2O2 can also decrease the ATP synthetic. ATP need by SERCA for catalizing the entry of Ca^{2+} to the ER depo, where 2 ions of Ca^{2+} require 1 ATP hydrolysis until the cytosolic Ca^{2+} is increased. (Hu et al., 1998; Moorena and Kinneb, 1998).

The administration of TG and CSA very significant decrease of Ca^{2+} basal occurred with $\Delta\sigma$decrease 23.30 Also combination of Ca^{2+} - free/EGTA , TG and CSA (picture 1 B,D) very significant decrease of cytosolic Ca^{2+} basal occurred with Δ 33.04. Even the work of TG is not as effective as H2O2 in competition to release Ca^{2+} from ER depo (Doan *et al*.,1994; Hu *et al*., 1998) until an excessive Ca^{2+} influx occurred from extracellular, but because of the entire blocking of SERCA pump, enables the power of CSA in decreasing the cytosolic Ca^{2+}, 2-4 times faster in taking Ca^{2+} by mitochondria (Rizzuto *et al.,* 1998, Csordás *et al*, 1999; Hajnocsky *et al*., 2000; Maili *et al*, 2003).

At the exposure of high glucose on the 9th day, picture 1 C, D and 2 show the Ca^{2+} influx from extracellular lower than 7th days But by the addition of TG, no significant decrease in cytosolic Ca^{2+} occurred. The accumulated H2O2 as a result of high glucose will directly cause the release of Ca^{2+} from the ER depo and restricting the work of TG at ER depo (Volk *et al*, 1997, Hu *et al*., 1998).

At the administration of a combination Ca^{2+} free/ EGTA and CSA increase non significantly in cytosolic Ca^{2+} basal Δ 3,11 compared to the administration of Ca^{2+}-free/EGTA only. This condition indicates that by adding CSA, the power of CSA in improving the intake of Ca^{2+} by mitochondria is slowing down. It is assumed that: (i) at a low cytosolic Ca^{2+} basal uniporter mitochondira could not take Ca^{2+} from cytosol. (Duncen, 2000; Crompton, 2000; Pozzan *and* Rizzuto, 2000, Smaili *et al*.; 2001; Brookes *et al*.,2004). (ii) in the absence of Ca^{2+} influx from extracellular (at a Ca^{2+} free/EGTA condition) will cause a decrease of Ca^{2+} concentration in cytosolic microdomain, thus increase the immediate intake of Ca^{2+} by mitochondria (Csordás *et al*., 1999; Hajnocsky *et al*., 2000). To test such assumption a combination of TG and CSA there will be a significant decrease of cytosolic Ca^{2+} basal with Δ 23,49. This condition tells the Ca $^{2+}$ influx from extracellular, will cause the taking of Ca^{2+} by mitochondria at cytosolic microdoman. This also occurred in the giving of a combination Ca^{2+}

free/EGTA, TG and CSA (picture 1 C, D) , a condition where a significant decrease in cytosolic Ca^{2+} basal occurred with Δ 7,78. TG as a blocker to SERCA pump may elevate the work of CSA in the intake of Ca^{2+} by mitochondria (Csordas *et al.,* 1999; Hajnoczky *et al*., 2000, Smaili *et al*.,2001).

Conclusion

On the IVth stage research a dynamic interaction change between ER organellas, extracellular and endothelial cells mitochondria at high glucosed exposure where:

(1) At a glucose exposure on the 3rd day, the administration of a combination TG and EGTA will casue an increase in cytosolic Ca^{2+}, TG triggers the improved release of Ca^{2+} from the depo and blocks the influx of Ca^{2+} from cytosol to the ER. as the main contribution to the increase of cytosolic Ca^{2+} through its work against SERCA and the intake of Ca^{2+} by mitochondria.

(2) On the 7th day of glucose exposure, the biggest contribution in increasing cytosolic Ca^{2+} derives from extracellular, so that an administration of EGTA may decrease the cytosolic Ca^{2+} in a significant way. Adding TG enables the elevation of the work of CSA in decreasing the cytosolic Ca^{2+} and increase the immediate intake of Ca^{2+} by mitochondria.

(3) On the glucose exposure on the 9th day the working power of CSA in elevating the intake of Ca^{2+} by mitochondria requires TG to act as blocker to the SERCA pump.

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