DOI: 10.1007/s10535-016-0612-8 BIOLOGIA PLANTARUM 60 (3): 595-600, 2016

BRIEF COMMUNICATION

Hydrogen sulfide as a signal molecule in hematin-induced heat tolerance of tobacco cell suspension

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Abstract

Carbon monoxide (CO) is considered as a new emerging cell signal molecule which is involved in plant growth, development, and acquisition of stress tolerance. In recent years, hydrogen sulfide (H_2S) has been found to have similar functions, but crosstalk between CO and H_2S in the acquisition of heat tolerance in plants is not clear. In this study, pretreatment of tobacco (*Nicotiana tabaccum* L.) cells cultured in a suspension with a CO donor hematin significantly increased survival percentage of cells under a heat stress and regrowth ability after the heat stress, alleviated a decrease in cell vitality, and accumulation of malondialdehyde. In addition, treatment with hematin enhanced the activity of L-cysteine desulfhydrase, a key enzyme in H_2S biosynthesis, which in turn induced accumulation of endogenous H_2S in tobacco cells. Interestingly, hematin-induced heat tolerance was enhanced by addition of NaHS, a H_2S donor, but weakened by specific inhibitors of H_2S biosynthesis DL-propargylglycine or its scavenger hypotaurine. Furthermore, pretreatment with hemoglobin (a CO scavenger) and zinc protoporphyrin IX (a CO specific synthetic inhibitor) had no significant effect on NaHS-induced heat tolerance of tobacco cells. These results suggest that CO pretreatment could improve the heat tolerance of tobacco suspension cultured cells, and H_2S might exert its signal role downstream to CO-induced heat tolerance.

Additional key words: carbon monoxide, high temperature, *Nicotiana tabaccum*, *in vitro* culture.

Carbon monooxide (CO) originates from heme degradation catalyzed by heme oxygenase which is a rate-limiting enzyme of heme degradation in plants (Maines 1997). Therefore, in pharmacological experiments, zinc proto-porphyrin IX (ZnPPIX) is widely used as specific CO synthetic inhibitor, and hematin and hemoglobin (Hb) as CO donor and scavenger, respectively (She and Song 2008).

Recently, CO is considered as a new emerging cell signal molecule with multiple functions (Maines 1997). It plays an important role in seed germination (Liu *et al.* 2010), growth and development regulation (Xuan *et al.* 2008), stomatal closure (She and Song 2008, Song *et al.* 2008), salt and heavy metal stress tolerance (Xie *et al.* 2008, Shen *et al.* 2011, Zhang *et al.* 2012, Zilli *et al.* 2014), and alleviation in oxidative stress (Sa *et al.* 2007). In addition, CO pretreatment improves the chilling tolerance of *Baccaurea ramiflora* (Bai *et al.* 2012). All the above mentioned processes involve crosstalk between CO and other signal molecules like NO, H_2O_2, and cyclic guanosine monophosphate (cGMP).

Like CO, hydrogen sulfide (H_2S) has long been known as phytotoxin which may be connected with the mass extinction of species in the Permian period (Li

Submitted 7 September 2015, *last revision* 14 December 2015, *accepted* 22 December 2015.
Abbreviations: cGMP - cyclic guanosine monophosphate; f.m. - fresh mass; Hb - hemoglobin; HO - heme oxygenase; HT - hypotaurine; LCD - L-cysteine desulfhydrase; MDA - malodialdehyde; MS - Murashige and Skoog; PAG - DL-propargylglycine; ROS - reactive oxygen species; ZnPPIX - zinc protoporphyrin IX.
Acknowledgements: This research was supported by the National Natural Science Foundation of China (31360057). We appreciate the reviewers and editors for their exceptionally helpful comments about the manuscript.
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2013, Lisjak *et al.* 2013, Calderwood and Kopriva 2014, Hancock and Whiteman 2014). Recently, H_2S has been considered as an endogenous gaseous signal molecule. Due to its dual role (a phytotoxin at a high concentration and a cell signal molecule at a low concentration), H_2S homeostasis in plant cells is very important. Hydrogen silfide homeostasis is mainly regulated by L-cysteine desulfhydrase (LCD, E.C. 4.4.1.1, responsible for H_2S release) and cysteine synthase (E.C. 4.2.99.8, H_2S is incorporated into O-acetyl-L-serine to form cysteine) (Lisjak*et al.* 2013) In pharmacological experiments, sodium hydrosulfide (NaHS) is commonly used as donor of H_2S, it releases H_2S when dissolved in water (Lisjak *et al.* 2013), whereas DL-pro-pargylglycine (PAG) is commonly used as specific inhibitor of H_2S biosynthesis, and hypotaurine (HT) as H_2S scavenger (Li *et al.* 2012b, Lisjak *et al.* 2013). Lately, many functions of H_2S have been uncovered, *e.g.*, in seed germination, organogenesis (Li *et al.* 2012a, Li and He 2015), stomata movements and osmotic stress (Christou *et al.* 2013), responses to salinity and heavy metals (Christou *et al.* 2013), chilling (Fu *et al.* 2013), and oxidative stress (Zhang *et al.* 2011). In maize and wheat seedlings, treatment with NaHS can improve heat tolerance (Li *et al.* 2013a,b, 2015c, Wu *et al.* 2013). Similarly, in tobacco suspension, NaHS increases the survival percentage under heat stress and the regrowth ability after heat stress (Li *et al.* 2012b, 2015a,b) implying that NaHS could improve the heat tolerance of tobacco cells, but whether this improvement is involved in a CO signal need to be further illustrated. In this study, the effect of pretreatment with hematin on heat tolerance of tobacco cell suspension cultured cells and involvement of H_2S were investigated. The purpose was to illustrate that CO could improve the heat tolerance of tobacco cells, and that this improvement involve a new downstream signal molecule H_2S.

Calluses were developed from the young stem pith of tobacco (*Nicotiana tabacum* L.) cv. Bright Yellow (Li *et al.* 2012b). Suspension cells derived from this callus were cultured in a liquid Murashige and Skoog (1962; MS) basal medium containing 0.9 μM 2,4-dichloro-phenoxyacetic acid and 0.4 μM kinetin in a rotary shaker at 120 rpm and 26 °C in the dark. The liquid medium was refreshed at weekly intervals, and the suspension cells were cultured according to Li *et al.* (2012b). At 5 d after subculture, the tobacco cells in the logarithmic phase of growth were collected and used for the following experiments.

To investigate the effects of pretreatment with a CO donor hematin on heat tolerance and involvement of H_2S, the 5-d-old tobacco cells were immediately transferred into hematin solutions with different concentrations [0 (control), 0.05, 0.10, 0.15, 0.20, 0.25, 0.30, and 0.35 μM]. The pH of the hematin stock solution was adjusted to 5.8 with KOH or HCl before being added to the cell cultures. All the cultures were grown on a rotary shaker at 120 rpm and 26 °C in the dark for 4 h, and then a half of the flasks were transferred to another rotary shaker at 120 rpm and 43 °C for 7 h to heat-stress the cultures (Li *et al.* 2012b). The remaining flasks were maintained on the rotary shaker at 26 °C for another 7 h. After 7 h, the cultures were harvested and the survival percentage of cells was determined as described below. All experiments were repeated at least three times and two replications in each time. After the heat stress of 43 °C for 7 h, the survival percentage of cells, regrowth ability, cell vitality, and malondialdehyde (MDA) content were determined as in previous experiments (Li *et al.* 2012b). During the treatment with 0.25 μM hematin, the cells were collected at various times (0, 2, 4, and 6 h), filtered, and washed with a fresh liquid MS basal medium without plant hormones. The activity of LCD and H_2S content were determined using previously described methods (Li 2015a,b).

To study the effect of pretreatment with a CO donor, scavenger, or inhibitor alone or in combination with a H_2S donor, scavenger, or inhibitor on heat tolerance of the tobacco cells, the 5-d-old tobacco cells were immediately transferred into different liquid MS media containing the following chemicals and treated for 4 h: *1*) 0 (control) or 0.25 μM hematin; *2*) 0.25 μM hematin + 0.5 mg dm^{-3} Hb as CO scavenger; *3*) 50 μM NaHS; *4*) 0.25 μM hematin +50 μM NaHS; *5*) 0.25 μM hematin + 50 μM HT as H_2S scavenger; *6*) 0.25 μM hematin + 50 μM PAG as H_2S biosynthesis inhibitor; *7*) 50 μM HT; *8*) 50 μM PAG; *9*) 50 μM NaHS + 0.5 mg dm^{-3} Hb; *10*) 50 μM NaHS + 0.5 μM ZnPPIX as CO biosynthesis inhibitor; *11*) 0.5 mg dm^{-3} Hb; and *12*) 0.5 μM ZnPPIX (for further information see Li *et al.* 2013a). The pH of all the solutions was adjusted to 6.0 with 1 M HCl or NaOH, and the pretreatments with different chemicals had no significant effect on cell survival in the normal culture conditions. After the heat stress, the survival percentage was calculated.

The experiment was set up according to a completely randomized design with five replications. Data were processed statistically using the software package *SPSS v. 21.0* (*SPSS*, Chicago, USA) based on the one-way analysis of variance, the significance of differences was analysed using Student's t-test. The figures were drawn by *SigmaPlot 12.5* (*Systat Software*, London, UK).

The application of hematin had no significant effects on the survival percentage of the suspension cells at the temperature of 26 °C when the concentrations were below 0.35 μM, but 0.35 μM hematin had a toxic effect on the cells of tobacco (Fig. 1*A*). It might be in relation to its strong affinity to a heme protein such as cytochrome oxidase (He and He 2014). Under the high temperature, however, the hematin pretreatments increased the survival percentage of the tobacco suspension cells. In particular, the treatment with 0.25 μM showed the most

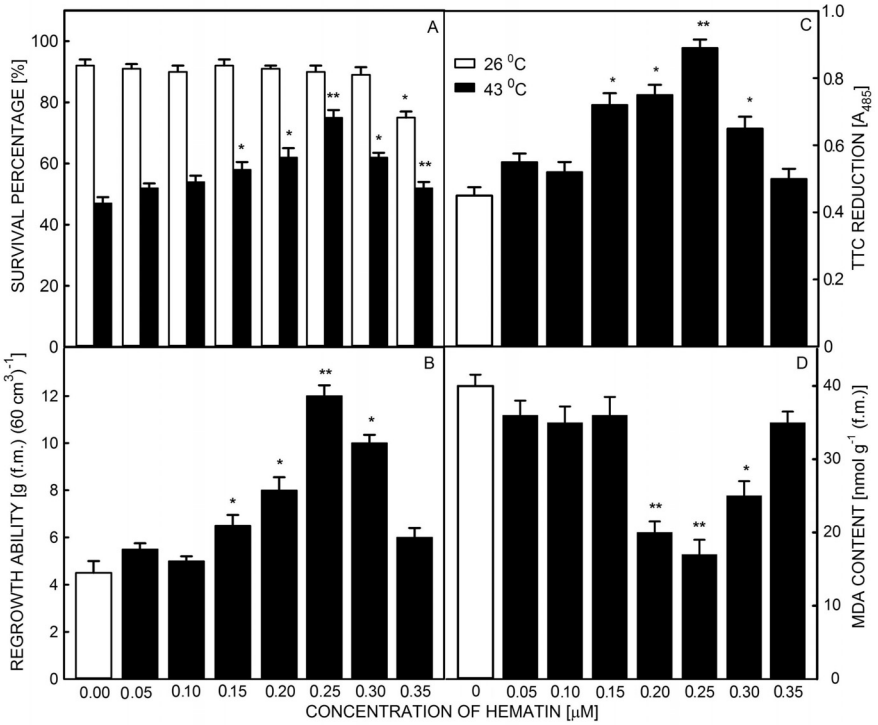

Fig. 1. Effects of hematin pretreatment on survival percentage (*A*), regrowth ability (*B*), cell vitality (*C*), and malondialdehyde (MDA) content (*D*) of a tobacco cell suspension under a heat stress at 43 °C. The 5-d-old suspension cells were subjected to heat stress for 7 h after being pretreated with 0 (control), 0.05, 0.10, 0.15, 0.20, 0.25, 0.30, or 0.35 μM hematin for 4 h. Means ± SEs of three experiments, * and ** indicate significant difference at $P < 0.05$ and $P < 0.01$, respectively, from the control.

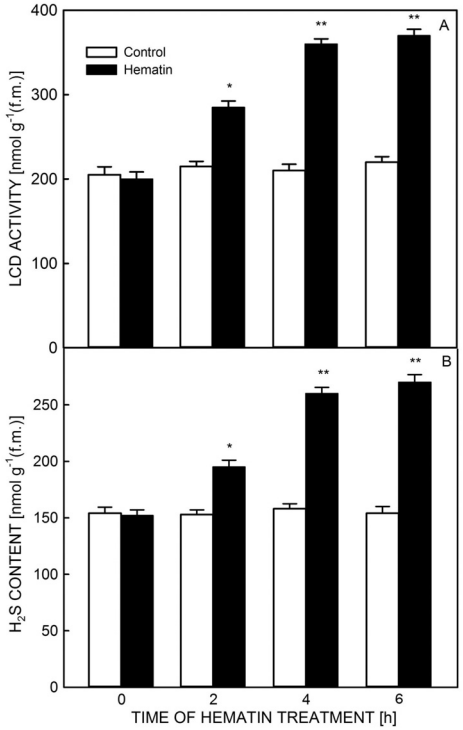

Fig. 2. Effects of hematin pretreatment on L-cysteine desulfhydrase (LCD) activity (*A*) and content of endogenous H_2S (*B*) in tobacco suspension cultured cells. The 5-d-old suspension cells were treated with 0 (control) or 0.25 μM hematin for 6 h, and then LCD activity and H_2S content of the tobacco cells determined every 2 h. Means ± SEs of three experiments, * and ** indicate significant difference at $P < 0.05$ and $P < 0.01$, respectively, from the control.

significant difference compared with the control ($P < 0.01$).

Therefore, 0.25 μM hematin was used in further experiments. In addition, pretreatments with hematin improved regrowth ability of the tobacco cells during recovery at 26 °C after the heat stress (Fig. 1*B*), alleviated a decrease in cell vitality (Fig. 1*C*), and reduced accumulation of MDA under the heat stress (Fig. 1*D*) compared with the control cells. Among the seven concentrations, the effect of 0.25 μM hematin was the most significant. The pretreatment with 0.25 μM hematin increased the activity of LCD and the content of H_2S in the tobacco cells, and this increase was enhanced with the increase of the treatment time; significant differences from control ($P < 0.01$) were found at 4 and 6 h of the treatment (Fig. 2*A*,*B*). These data suggest that pretreatment with CO donor hematin could improve the heat tolerance of the tobacco suspension cultured cells in a concentration-dependent manner and increase the content of H_2S by activating LCD.

Further, the 5-d-old tobacco cells were treated with 0.25 μM hematin alone or in combination with Hb, NaHS, HT, or PAG for 4 h, and then subjected to a heat stress at 43 °C for 7 h. The pretreatment with 0.25 μM hematin improved the survival percentage of the tobacco cells (Fig. 1*A*, Table 1), and this improvement was enhanced by application of 50 μM NaHS but weakened by the inhibitor of H_2S biosynthesis PAG as well as by the H_2S scavenger HT (Table 1). In addition, the H_2S biosynthesis inhibitor and the H_2S scavenger alone significantly reduced the survival percentage of the cells under the heat stress compared with the control (Table 1), further suggesting the importance of H_2S in the acquisition of heat tolerance in the tobacco cells. These results imply that H_2S exerts its role as downstream signal

Table 1. Effects of 4-h pretreatments with hematin, hemoglobin (Hb), H_2S donor NaHS, H_2S scavenger hypotaurine (HT), and H_2S biosynthesis inhibitor DL-propargylglycine (PAG) on a survival percentage of tobacco cells after a heat stress. Means ± SEs of three experiments, * and ** indicate significant differences from the control at $P < 0.05$ and $P < 0.01$, respectively.

Treatments	Survival percentage [%]
Control	48 ± 2.5
Hematin (0.25 μM)	75 ± 3.4**
Hematin + Hb (0.5 mg dm^{-3})	51 ± 1.3
NaHS (50 μM)	78 ± 4.1**
Hematin + NaHS	85 ± 3.7**
Hematin + HT (50 μM)	62 ± 3.5*
Hematin + PAG (50 μM)	65 ± 3.2*
HT	35 ± 2.1*
PAG	38 ± 3.0*

Table 2. Effects of pretreatment with CO donor hematin, CO scavenger hemoglobin (Hb), and CO biosynthesis inhibitor zinc protoporphyrin IX (ZnPPIX) on NaHS-induced heat tolerance of tobacco cell suspensions measured as a survival percentage of cells after a 4-h heat stress. Means ± SEs of three experiments, ** indicate significant differences from the control at $P < 0.01$.

Treatments	Survival percentage [%]
Control	48 ± 2.5
NaHS (50 μM)	78 ± 4.1**
NaHS + Hb (0.5 mg dm^{-3})	77 ± 3.3**
NaHS + ZnPPIX (50 μM)	75 ± 2.4**
Hb	43 ± 2.6
ZnPPIX	40 ± 3.20

of CO in hematin-induced heat tolerance in the tobacco cells.

Our previous (Li *et al.* 2012b, 2015a,b) and present (Tables 1, 2) results show that pretreatment with NaHS could increase the heat tolerance of the tobacco suspension cultured cells. To further study the effect of a CO scavenger and a biosynthesis inhibitor on NaHS-induced heat tolerance in the tobacco cells, the 5-d-old cells were treated with 50 μM NaHS alone or in combination with the CO scavenger Hb or the biosynthesis inhibitor ZnPPIX for 4 h, and then were subjected to the heat stress at 43 °C for 4 h (Table 2). The treatment with 50 μM NaHS increased the survival percentage of the tobacco cells, and this increase was not weakened by application of 0.5 mg dm^{-3} Hb or 0.5 μM ZnPPIX, whereas Hb or ZnPPIX alone slightly reduced the survival percentage of the tobacco cells compared with the control. All of the above-mentioned results indicate that CO might exert its role upstream to H_2S in the acquisition of heat tolerance.

In plant cells, survival percentage, cell vitality, MDA content and regrowth ability are commonly used as heat tolerance indexes (Ishikawa *et al.* 1995, Li *et al.* 2012b). In the present study, pretreatment with low concentrations of the CO donor hematin (0.15, 0.20, 0.25, and 0.30 μM) could improve the heat tolerance of the tobacco suspension (Fig. 1). On the contrary, the high concentration of hematin (0.35 μM) declined the survival percentage of the tobacco cells under both non-stress conditions and heat stress conditions (Fig. 1*A*) indicating that the high concentration of CO had a toxic effect on the tobacco cells, similarly to other second messengers like Ca^{2+}, H_2O_2, H_2S, and NO. Similarly, CO as a second messenger plays a very important role in alleviation from oxidative stress (Sa *et al.* 2007), salt stress (Xie *et al.* 2008, Zhang *et al.* 2012, Zilli *et al.* 2014), heavy metal stress (Shen *et al.* 2011), and chilling stress (Bai *et al.* 2012), as well as these alleviative effects of H_2S are involved in many downstream signal molecules of CO

such as NO, H_2O_2, and cGMP. However, whether the acquisition of heat tolerance induced by CO was also implicated in H_2S signal needs to be further illustrated.

As mentioned above, LCD is a rate-limiting enzyme of H_2S biosynthesis in plants (Lisjak *et al.* 2013). In alfalfa seedlings, NaCl stress causes growth inhibition and lipid peroxidation, but negative effects of NaCl are alleviated by NaHS, whereas aggravated by PAG and HT, respectively (Lai *et al.* 2014). In addition, NaCl stress also increases the total LCD activity and content of endogenous H_2S (Lai *et al.* 2014), which may be a second messenger involved in salt tolerance. Fu *et al.* (2013) have found that chilling pretreatment enhances H_2S content by activating LCD activity and gene expression in *Vitis vinifera*, which in turn improves the subsequent chilling tolerance. In addition, H_2S pretreatment also shows a similar effect (Fu *et al.* 2013). In maize seedlings, salicylic acid pretreatment significantly increases LCD activity followed by increasing H_2S content, which in turn improves the heat tolerance of maize seedlings (Li *et al.* 2015c) suggesting that the heat tolerance induced by salicylic acid in plants might be achieved by the downstream signal molecule H_2S. Similarly in the present study, treatment with hematin improved the activity of LCD in the tobacco cells (Fig. 2*A*), which in turn elevated the content of H_2S (Fig. 2*B*) and ultimately improved the heat tolerance of the tobacco cells (Fig. 1, Table 1) implying that the pretreatment with CO could increase the accumulation of endogenous H_2S in the tobacco cells by activating LCD activity, and this accumulation might be involved in heat tolerance induced by hematin.

NaHS, PAG, and HT are commonly used as donor, specific inhibitor, and scavenger, respectively, of H_2S (Li *et al.* 2012b, Lisjak *et al.* 2013). In strawberry seedlings, Christou *et al.* (2014) have found that hydroponic pretreatment with NaHS enhances the heat tolerance, and alleviates the accumulation of MDA, H_2O_2, and NO. Our previous results also identified that NaHS pretreatment can improve heat tolerance of tobacco cells (Li *et al.* 2012b, 2015a). In this study, we illustrate that the accumulation of endogenous H_2S in the tobacco cells was implicated in heat tolerance because heat tolerance was induced by hematin alone, further improved by NaHS, but weakened by HT or PAG (Table 1). In addition, the NaHS treatment also increased the heat tolerance of the tobacco cells (Li *et al.* 2012b, Tables. 1, 2), but a significant difference was not observed in the tobacco cells treated with NaHS in combination with Hb and ZnPPIX, respectively. These data indicate that CO treatment could improve the heat tolerance of the tobacco cells, and the acquisition of heat tolerance induced by CO might be achieved by its downstream signal molecule H_2S.

In summary, the pretreatment of the tobacco cells with different concentrations of the CO donor hematin significantly increased the heat tolerance of the cells. In addition, the hematin treatment stimulated an increase in LCD activity and endogenous H_2S accumulation in the tobacco cells. Also, hematin-induced heat tolerance was enhanced by NaHS, weakened by PAG and HT, whereas the Hb and ZnPPIX treatments alone did not significantly affect NaHS-induced heat tolerance in the tobacco cells. These results imply that hematin pretreatment could improve heat tolerance of the tobacco cells, and H_2S might function as downstream signal molecule of CO, but the precise physiological, biochemical, and molecular mechanisms need future research.

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