Available online at www.sciencedirect.com
SciVerse ScienceDirect
EJSO
the Journal of Cancer Surgery
ELSEVIER
EJSO 38 (2012) 143–149
www.ejso.com

Phase II trial of preoperative S-1 plus cisplatin followed by surgery for initially unresectable locally advanced gastric cancer

K. Inoue [a,*], Y. Nakane [a], M. Kogire [b], K. Fujitani [c], Y. Kimura [d], H. Imamura [e], S. Tamura [f], S. Okano [g], A.H. Kwon [a], Y. Kurokawa [h], T. Shimokawa [i], H. Takiuchi [j], T. Tsujinaka [c], H. Furukawa [e]

[a] *Department of Surgery, Kansai Medical University, Shinmachi 2-3-1, Hirakata city, Osaka 573-1191, Japan*
[b] *Department of Surgery, Kishiwada City Hospital, Osaka, Japan*
[c] *Department of Surgery, National Hospital Organization Osaka National Hospital, Osaka, Japan*
[d] *Department of Surgery, NTT West Osaka Hospital, Osaka, Japan*
[e] *Department of Surgery, Sakai Municipal Hospital, Osaka, Japan*
[f] *Department of Surgery, Kansai Rosai Hospital, Hyogo, Japan*
[g] *Department of Surgery, Matsushita Memorial Hospital, Osaka, Japan*
[h] *Department of Gastroenterological Surgery, Graduate School of Medicine, Osaka University, Osaka, Japan*
[i] *Graduate School of Medicine and Engineering, University of Yamanashi, Yamanashi, Japan*
[j] *2nd Department of Internal Medicine, Osaka Medical College, Osaka, Japan*

Accepted 21 November 2011
Available online 9 December 2011

Abstract

Background: The aim of this study was to evaluate the efficacy and feasibility of preoperative chemotherapy with S-1 plus cisplatin in patients with initially unresectable locally advanced gastric cancer.
Methods: We enrolled patients with initially unresectable locally advanced gastric cancer because of severe lymph node metastases or invasion of adjacent structures. Preoperative chemotherapy consisted of S-1 at 80 mg/m^2 divided in two daily doses for 21 days and cisplatin at 60 mg/m^2 intravenously on day 8, repeated every 35 days. If a tumor decreased in size, patients received 1 or 2 more courses. Surgery involved radical resection with D2 lymphadenectomy.
Results: Between December 2000 and December 2007, 27 patients were enrolled on the study. No CR was obtained, but PR was seen in 17 cases, and the response rate was 63.0%. Thirteen patients (48.1%) had R0 resections. There were no treatment related deaths. The median overall survival time (MST) and the 3-year overall survival (OS) of all patients were 31.4 months and 31.0%, respectively. Among the 13 patients who underwent curative resection, the median disease-free survival (DFS) and the 3-year DFS were 17.4 months and 23.1%, respectively. The MST and the 3-year OS were 50.1 months and 53.8%, respectively. The most common site of initial recurrence after the R0 resection was the para-aortic lymph nodes.
Conclusions: Preoperative S-1 plus cisplatin can be safely delivered to patients undergoing radical gastrectomy. This regimen is promising as neoadjuvant chemotherapy for resectable gastric cancer. For initially unresectable locally advanced gastric cancer, new trials using more effective regimens along with extended lymph node dissection are necessary.

Keywords: Neoadjuvant chemotherapy; Lymph node dissection; Bulky lymph node; TS-1; Cisplatin; Para-aortic lymph node

Introduction

Gastric cancer is still one of the most common cancers in the world; 876,000 new cases were anticipated worldwide in the year 2000.[1] In Japan, 110,323 new cases were anticipated in the year 2003 and the 5-year survival rate of gastric cancer diagnosed from 1993 to 1996 was 54.4%.[2,3]

Currently, surgery remains the mainstay of curative treatment. However, only an R0 resection is associated with significant cure rates. Patients having microscopic (R1) or macroscopic (R2) residual tumor have an extremely poor prognosis.[4]

* Corresponding author. Tel./fax: +81 72 804 2865.
E-mail address: inoueke@hirakata.kmu.ac.jp (K. Inoue).

0748-7983/$ - see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.ejso.2011.11.009

Preoperative and neoadjuvant chemotherapy represent investigational options. The rationale of preoperative chemotherapy is based on the difficulty of performing an R0 resection in patients with initially unresectable locally advanced tumors and the high risk of micrometastatic disease in these patients. Neoadjuvant chemotherapy has potential for resectable gastric cancer for the purpose of treating micrometastases.

Intensive chemotherapy is necessary for the improvement of the R0 resection rate and complete elimination of the micrometastases. However, it is difficult for patients who undergo gastrectomy to tolerate intensive chemotherapy. Because weight decreases by gastrectomy, it is necessary to reduce the dose of chemotherapy. The tolerance to chemotherapeutic agents with digestive organ toxicity was often reduced by gastrectomy-related gastrointestinal effects.

S-1 (TS-1, Taiho Pharmaceutical, Tokyo, Japan) is an orally active combination of tegafur (a prodrug that is converted by cells to fluorouracil), gimeracil (an inhibitor of dihydropyrimidine dehydrogenase, which degrades fluorouracil), and oteracil (which inhibits the phosphorylation of fluorouracil in the gastrointestinal tract, thereby reducing the gastrointestinal toxic effects of fluorouracil) at a molar ratio of 1:0.4:1. The response rate of S-1 alone exceeded 40% in two phase 2 trials involving patients with metastatic gastric cancer.[5,6] The combination chemotherapy of S-1 plus cisplatin (CDDP) achieved a high response rate (74%, 95%CI: 54.9–90.6) in a previous phase I/II study of patients with metastatic gastric cancer.[7]

These factors led us to perform the current phase II trial to investigate the use of an active preoperative chemotherapy regimen. The primary objectives of the trial were to investigate tolerance to the preoperative regimen, its effects on operative morbidity and mortality, and the response rate. Secondary objectives included evaluation of the R0 resection rate, disease-free and overall survival, and failure pattern.

Patients and methods

Patients

The study was conducted as a prospective multi-institutional phase II trial by the Osaka Gastrointestinal Cancer Chemotherapy Study Group (OGSG) in Japan. All patients had histologically confirmed adenocarcinoma of the stomach. They also had to have initially unresectable locally advanced tumors because of invasion to adjacent structures or severe lymph node metastases, staged by contrast-enhanced CT as T2-3N2-3M0 or T4NanyM0, according to the Japanese Classification of Gastric Carcinoma (2nd English Edition).[8] They also had to have lymph node metastases that were measurable according to the RECIST[1.0] guidelines.[9] We did not require laparoscopic staging as an entry criterion for this study. Any sites of suspected M1 disease had to be ruled out prior to entrance into the study. No prior chemotherapy or radiation was allowed. The age range was 20–75 years. The performance status (ECOG) was 0 from 1.

Because of the worse prognosis of type IV gastric cancer, also known as scirrhous or linitis plastica, we excluded such cases.[10] Acceptable hematologic profile (WBC $\geqq$ 4000 cells/mm^3, hemoglobin $\geqq$ 8.0 g/dl, platelets $\geqq$ 100,000 cells/mm^3), and renal (BUN $\leqq$ 25 mg/dl, creatinine $\leqq$ 1.2 mg/dl and/or creatinine clearance > 60 ml/min) and hepatic function (total serum bilirubin < 1.5 mg/dl) were required. In addition, certain respiratory function test results (ratio of the forced expiratory volume in one second $\geqq$ 50%, PaO2 in room air $\geqq$ 70 mmHg) were required criteria. No clinically significant auditory impairment was allowed. Patients with prior cancer diagnosed during the previous 5-year period (except for colon carcinoma *in situ*) were excluded. Other exclusion criteria included significant cardiac disease, pregnancy or serious infections. The protocol was reviewed and approved by the Institutional Review Board of each institution. All patients gave written informed consent.

Preoperative chemotherapy

Patients found to have locally advanced gastric cancer as defined above, received two cycles of S-1 plus cisplatin every 35 days. Preoperative chemotherapy consisted of S-1 at 80 mg/m^2 divided in two daily doses for 21 days and cisplatin at 60 mg/m^2 intravenously on day 8. Physical examination, abdominal CT scan and assessment of toxicity were performed prior to each cycle. The response measurement of the preoperative chemotherapy was carried out according to the RECIST[1.0] guidelines. Because it was preoperative chemotherapy, response was not confirmed at least 4 weeks apart. Toxicity was recorded and graded according to the National Cancer Institution Common Toxicity Criteria (NCI-CTC) version 2.0 scale. Operative complication was graded according to the Common Terminology Criteria for Adverse Events v4.0 (CTCAE v4.0). If a tumor decreased in size, according to protocol criteria, we added 1 or 2 more courses. If curative resection was considered possible after planned chemotherapy, the patient had surgery. If curative resection was considered difficult, a further course of chemotherapy was added. The doses of both agents were attenuated for grade $\geqq$3 toxicities, using standard reduction criteria.

Surgery

The surgery was planned for 3–6 weeks from the day of last administration of chemotherapy. Surgery involved a radical resection, the extent of which (total or distal gastrectomy) depended on the site of the primary tumor, with a D2 lymphadenectomy. We performed D2 or more dissection in patients with metastasis to N3 lymph nodes before chemotherapy. Spleen preservation in total gastrectomy procedure was entrusted to the decision of each clinician.

Patients in whom curative resection was impossible underwent palliative operation. The postoperative treatment was left to the decision of each physician.

Biostatistical considerations

The 3 primary end points of the study were as follows; 1) tolerance to preoperative chemotherapy, 2) operative morbidity and mortality, and 3) objective response rate (ORR). Secondary end points were R0 resection rate, failure pattern, and disease-free and overall survival. One of the primary end points was ORR. The number of patients to be enrolled was calculated at 24, which was required given the assumption that the 95% confidence interval (CI) would be ±20%, assuming an expected response rate of 60%. Finally, we set the number as 30 patients in consideration of disqualified patients. The early stopping criterion of the trial was 3 treatment related deaths. Analogous samples were used to estimate the response rate, R0 resection rate, operative morbidity and mortality, and incidence of treatment related grade 3–4 toxicity. Overall survival (OS) of all patients was calculated from the day of registration in the trial. OS and disease-free survival (DFS) of the patients who underwent R0 resections were calculated from the day of surgery. Survival distributions were estimated using the Kaplan–Meier method.

Follow-up

Following completion of chemotherapy and surgery, patients were followed at 3- monthly intervals until year 3. Thereafter, 6-month follow-up visits were performed. CT scans and appropriate blood studies were performed on the occasion of each evaluation.

Results

Patient population

Between December 2000 and December 2007, 27 patients with initially unresectable local advanced gastric cancer were enrolled into the study from 9 institutions. As shown in Table 1, the male to female ratio was 20:7. The median age was 63 years. As for the histologic type, 15 cases were undifferentiated (including signet ring cell carcinoma) and 11 cases were differentiated type. One case was classified as mucinous carcinoma. There were 3 cStage IIIa (11.1%) preoperatively, 8 cStage IIIb (29.6%), and 16 cStage IV (59.3%).

Preoperative chemotherapy

The median number of preoperative chemotherapy regimens was 3 courses. Grade 3–4 toxicities associated with preoperative S-1/CDDP are described in Table 2. Hematologic toxicity (Grade 3/4) was 7.4% and non-hematologic toxicity (Grade 3/4) was 3.7%. Treatment was generally well tolerated and no chemotherapy-related deaths were observed. While there was no CR, there were 17 cases of PR and the response rate was 63.0% [95%CI: 42.4–80.6] (Table 2).

Table 1
Patient characteristics ($n = 27$).

		Number	%
Age, years	Median (range)	63	(48–75)
Gender	Male	20	74.1
	Female	7	25.9
Histology	Differentiated	11	40.7
	Undifferentiated	15	55.6
	Other	1	3.7
Pretreatment cStage	T2N2M0 (IIIA)	3	11.1
	T3N2M0 (IIIB)	7	25.9
	T4N1M0 (IIIB)	1	3.7
	T2N3M0 (IV)	5	18.5
	T3N3M0 (IV)	6	22.2
	T4N2M0 (IV)	3	11.1
	T4N3M0 (IV)	2	7.4

Operative outcome

All patients who were entered into this trial had initially unresectable tumors. Nine patients were diagnosed as being unresectable when chemotherapy was completed and did not undergo surgery. Eighteen patients (66.7%) underwent laparotomy (Table 3). Thirteen patients (48.1%) had R0 resections. Three patients (11.1%) underwent R1 surgery, because of positive results of peritoneal washing cytology. Two patients underwent simple laparotomy because of peritoneal metastases or unresectable local extension of metastatic lymph nodes. Postoperative complications are described in Table 3. The incidence of complications was 22.2%. One patient underwent operative intervention because of pancreatic leakage; however, there were no surgery-related deaths.

Table 2
Courses, responses and toxicities of preoperative chemotherapy.

				n	%
Courses	Median (range)			3	(1–9)
Response	CR			0	0.0
	PR			17	63.0
	SD			6	22.2
	PD			4	14.8
Toxicities		Grade1/2		Grade3/4	
		n	%	*n*	%
	Neutropenia	10	37.0	2	7.4
	Thrombocytopenia	3	11.1	1	3.7
	Hemoglobin	21	77.8	1	3.7
	Vomiting	7	25.9	1	3.7
	Nausea	13	48.1	1	3.7
	Diarrhea	4	14.8	1	3.7
	Anorexia	17	63.0	1	3.7
	Cerebral infarction	0	0	1	3.7
Treatment related death				0	0.0

Table 3
Operative outcome ($n = 27$).

		Number	%
No operation		9	33.3
Operation		18	66.7
	R0 resection	13	48.1
	R1 resection	3	11.1
	R2 resection	0	0
	Simple Laparotomy	2	22.2
Complications			
	None	14	77.8
	Pancreatic leak	3 (Grade 1: 2, Grade 4: 1)	16.7
	Lymphorrhea	1 (Grade 1)	5.6
	Anastomotic leak	0	0.0
Re-operation		1	5.6
Mortality		0	0.0

Seven of 9 patients who did not undergo surgery received 2nd-line chemotherapy (S-1: 3 patients, S-1/CPT-11: 2 patients, CPT-11/CDDP: 1 patient, Paclitaxel: 1 patient). Four of 5 patients who underwent R1-2 surgery received further chemotherapy (S-1/Paclitaxel: 2 patients, S-1: 1 patient, CPT-11/CDDP: 1 patient).

Overall survival of all patients

Only one patient was lost to follow-up at 8 months from the first day of preoperative chemotherapy, but all other patients were followed more than three years. The median overall survival time and the 3-year overall survival rate of all patients were 31.4 months and 31.0% [95%CI: 17.5–55.1], respectively.

DFS, OS, and first relapse site of patients who underwent R0 resection

Thirteen patients underwent R0 resection. The details of these patients are shown in Table 4. Twelve of these 13 patients (92.3%) achieved PR after preoperative chemotherapy. The median number of course of chemotherapy of these patients was 3 (2–5). Of these patients, only 2 patients (15.4%) underwent D2 plus para-aortic lymph node dissection (D3). Downstaging was observed in 11 patients (84.6%). Seven of 13 patients received postoperative adjuvant chemotherapy (S-1: 4 patients, S-1 plus CDDP: 1 patient, CPT-11: 1 patient, CPT-11/CDDP: 1 patient). To date, recurrence has been diagnosed in 10 patients. First relapse site of five of ten patients was para-aortic lymph nodes. The median disease-free survival time and the 3-year disease-free survival rate of the 13 patients were 17.4 months and 23.1% [95%CI: 8.6–62.3], respectively (Fig. 1A). The median overall survival time and the 3-year overall survival rate of the 13 patients were 50.1 months and 53.8% [95% CI: 32.6–89.1], respectively (Fig. 1B).

Discussion

The combination chemotherapy of S-1 plus cisplatin was chosen because it had achieved a high response rate of 74% (95%CI: 54.9–90.6) in previous phase I/II study of patients with metastatic gastric cancer. The incidences of severe (Grade 3/4) hematological and non-hematological toxicities were 15.8 and 26.3%, respectively.[7] A randomized controlled trial in Japan showed the superiority of S-1/cisplatin compared with S-1 monotherapy according to the response rate and survival for metastatic gastric cancer.[11] Therefore, S-1/cisplatin therapy is now the standard treatment for metastatic gastric cancer in Japan.

This multi-institutional phase II prospective trial of preoperative chemotherapy in initially unresectable locally advanced gastric cancer showed that preoperative chemotherapy using S-1/cisplatin was not only feasible but also achieved a high response rate. The overall response rate was 63.0% [95%CI: 42.4–80.6]. The incidence of grade 3/4 toxicities was less than 10% and treatment related

Table 4
Patients who underwent R0 resection.

No.	cStage	Course	Response	Gastrectomy	D	Combined resection	fStage	Nodes	First relapse
1	T3N2M0 (IIIB)	2	PR	Distal	D3	Liver, Gallbladder	T2N2M0 (IIIA)	4	None
2	T3N3M0 (IV)	3	PR	Total	D2	Spleen, Panc. (tail) Gallbladder	T2N2M0 (IIIA)	6	Brain
3	T3N2M0 (IIIB)	2	PR	Total	D2	Spleen	T2N2M0 (IIIA)	10	Lymph (para AO)
4	T3N2M0 (IIIB)	2	PR	Distal	D3	None	T2N2M0 (IIIA)	3	None
5	T3N2M0 (IIIB)	3	PR	Total	D1*	Liver	T2N0M0 (IB)	0	None
6	T2N2M0 (IIIA)	2	SD	Distal	D2	Panc. (head)	T4N3M0 (IV)	7	Peritoneum
7	T4N2M0 (IV)	3	PR	Total	D2	Spleen, Panc. (tail)	T3N2M0 (IIIB)	10	Lymph (para AO)
8	T2N3M0 (IV)	4	PR	Distal	D2	Gallbladder	T2N2M0 (IIIA)	1	Bone
9	T4N3M0 (IV)	3	PR	Distal	D2	None	T1N0M0 (IA)	0	Lung
10	T4N1M0 (IIIB)	3	PR	Total	D2	Spleen	T2N2M0 (IIIA)	4	Lymph (hepatic)
11	T2N3M0 (IV)	5	PR	Total	D1*	None	T2N3M0 (IV)	2	Lymph (para AO)
12	T2N2M0 (IIIA)	3	PR	Total	D1*	None	T2N0M0 (IB)	0	Lymph (para AO)
13	T3N2M0 (IIIB)	3	PR	Total	D1*	None	T2N2M0 (IIIA)	13	Lymph (para AO)

D1*: we performed almost D2 dissection, but it classified D1 dissection according to the Japanese classification of gastric carcinoma (2nd English edition), because of preserving spleen.

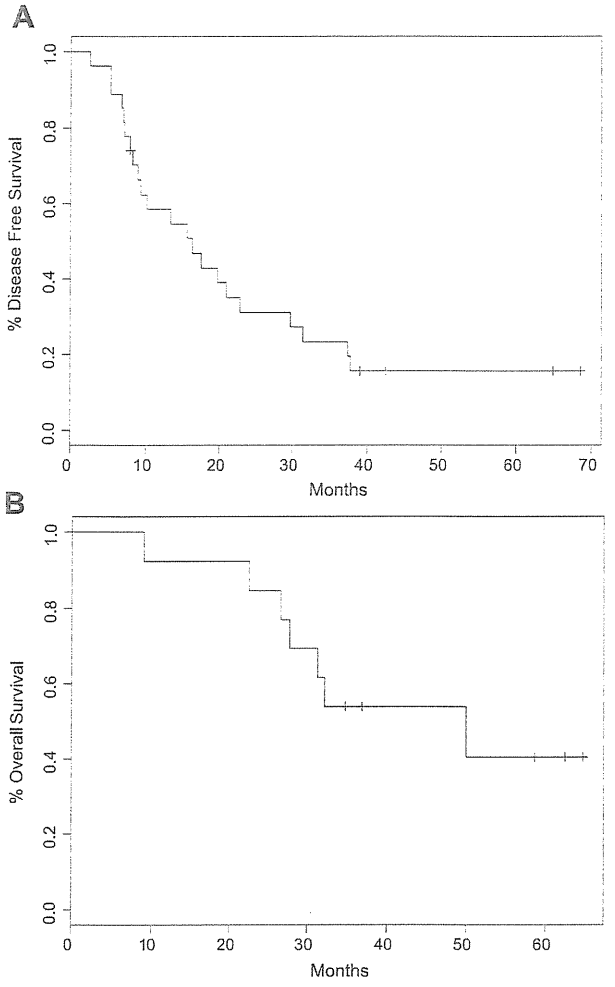

Figure 1. Disease-free and overall survival of the patients who underwent R0 surgery ($n = 13$).

mortality was 0.0%. Similar results were reported in other studies.[12,13] These results encourage the use of S-1/cisplatin combination chemotherapy as neoadjuvant treatment for patients who have resectable gastric cancer. Such trials are currently under way in Japan.[14,15]

The recently completed MAGIC trial constitutes a larger study regarding neoadjuvant chemotherapy in gastric cancer. In this study, 503 patients were randomized to three cycles of pre- and three cycles of postoperative epirubicin/cisplatin/5-FU (ECF) chemotherapy or surgery alone. Neoadjuvant chemotherapy was tolerable and was completed in 88% of patients. Significant downsizing (5.0 versus 3.1 cm median tumor size, $P < 0.001$), downstaging (54% versus 36% T1–T2 tumors, $P = 0.01$) and enhanced resectability (79% versus 69%, $P = 0.02$) were noted. Improved progression-free survival and survival were demonstrated, with an overall 5-year survival of 36% versus 23% for those undergoing surgery alone.[16] We should conduct phase III clinical trials of the neoadjuvant chemotherapy of S-1/cisplatin therapy for resectable gastric cancer.

In Japan, the ACTS-GC trail demonstrated a survival advantage of postoperative adjuvant chemotherapy after R0 resection. R0 patients were randomized to adjuvant chemotherapy using S-1 (529 patients) versus surgery alone (530 patients); improved survival (3-year overall survival rates of 80.1% versus 70.1%, $P = 0.003$) was noted.[17] Adjuvant chemotherapy, as reported by the ACTS-GC Group, is now considered a standard treatment for R0 patients. However, of the 283 patients who had stage III disease and received S-1 adjuvant chemotherapy, 73 patients died. The hazard ratio of the adjuvant chemotherapy group worsened with an increasingly advanced stage. These results suggest that S-1 monotherapy is insufficient for patients who have stage III or more. However, for patients who have initially unresectable gastric cancer like the patients enrolled in this trial, S-1/cisplatin chemotherapy is insufficient because of the high relapse rate of patients who underwent R0 resection.

For the patients immediately after gastrectomy, highly toxic chemotherapy is difficult because of overlaps between chemotherapy-induced gastrointestinal toxicity and digestive symptoms due to gastrectomy.[18] Therefore, further improvements in preoperative therapy will require development of more effective chemotherapeutic regimens. During the last decade, several new agents with promising activity against gastric cancer were identified. These include paclitaxel, docetaxel, irinotecan and trastuzumab. These agents are now undergoing phase II and III trials, as part of combination regimens.[19–22] If improved outcome is seen in metastatic disease, these agents will undergo extensive testing in the preoperative setting.

The absence of laparoscopic staging might have allowed inclusion of patients with positive peritoneal cytology or small peritoneal implants that could have disappeared with the chemotherapy; these patients have a worse prognosis, which could have impacted on the final results. Actually, there were 3 cases of positive cytology at exploration after chemotherapy. Laparoscopic staging should be mandatorily included in future similar projects.

An interesting point is that there were many para-aortic lymph node recurrences in the patients who underwent R0 resection. Among 13 patients who underwent curative resection, initial recurrence in 5 patients was in a para-aortic lymph node. These patients had not undergone para-aortic lymph node dissection. The prognostic improvement effect of the para-aortic lymph node dissection was refuted by two clinical trials.[23,24] In the JCOG 9501 trial, 523 patients with resectable gastric cancer were enrolled, and 263 were assigned to D2 group and 260 were assigned to D2 plus para-aortic nodal dissection. The 5-year overall survival rate was 69.2% for D2 lymphadenectomy group and 70.3% for the D2 lymphadenectomy plus para-aortic nodal dissection group; the hazard ratio for death was 1.03 (95%CI, 0.77 to 1.37; $P = 0.85$). There were also no significant differences in recurrence-free

survival and the pattern of recurrence between the two groups.[23] In the East Asian Surgical Oncology Group trial, 269 patients with resectable gastric cancer were enrolled, and 135 were assigned to the D2 group and 134 were assigned to the D2 plus para-aortic nodal dissection. The 5-year overall survival rates were 52.6% for the D2 lymphadenectomy group and 55.0% for the D2 lymphadenectomy plus para-aortic nodal dissection group. There was no significant difference in survival between the two groups ($P = 0.801$).[24] It was concluded that the D2 lymphadenectomy plus para-aortic nodal dissection did not improve prognosis regarding D2 lymph node dissection in the resectable gastric cancer.

However, in these trials, patients who had gross metastases to the para-aortic nodes were excluded. The incidence of metastases in the para-aortic nodes was lower than expected in 8.5% and 9.7%, respectively. The median number of metastatic nodes was only 2 nodes among the patients who underwent D2 plus para-aortic nodal dissection in the JCOG 9501. In the East Asian Surgical Oncology Group trial, the mean number of metastatic nodes was 5.9 in the para-aortic lymph node dissection group.

Recently, 15-year follow-up results of a randomized nationwide Dutch D1D2 trial were published. 711 patients underwent randomly assigned treatment with curative intent (380 in the D1 group and 331 in the D2 group). Overall 15-year survival was 21% for the D1 group and 29% for the D2 group. Gastric cancer-related death rate was significantly higher in the D1 group (48%, 182 patients) than that in the D2 group (37%, 123 patients). Local recurrence was 22% (82 patients) in the D1 group versus 12% (40 patients) in D2, and regional recurrence was 19% (73 patients) in D1 versus 13% (43 patients) in D2. After a median follow-up of 15 years, D2 lymphadenectomy was associated with lower locoregional recurrence and gastric cancer-related death rates than D1 surgery.[25] This difference was greater in the patients with lymph node metastases from 7 to 15.[26]

The observation period was shorter in the clinical trials of JCOG and East Asian Surgical Oncology Group than in the Dutch trail, and fewer mortality events occurred and also fewer metastases to lymph nodes. Therefore, para-aortic lymph node dissection might have better prognosis in patients with severe lymph node metastases like the patients enrolled in our trial.

In summary, preoperative S-1/cisplatin can be safely delivered to patients undergoing radical gastrectomy. The response rate was high, with no increase in operative morbidity and mortality compared with those upon surgery without preoperative chemotherapy.[27] Controlled trials of neoadjuvant chemotherapy using this regimen with the postoperative S-1 monotherapy for resectable gastric cancer are necessary. For initially unresectable locally advanced gastric cancer, the rate of recurrence was high, and the most common initial recurrent site was para-aortic lymph node. New trials, using a more effective regimen along with extended lymph node dissection are necessary.

Conflict of interest statement

The authors declare no conflict of interest.

Acknowledgments

The authors thank the members of the OGSG data center and operations office. The authors are also indebted to Prof. J. Patrick Barron, Chairman of the Department of International Medical Communications of Tokyo Medical University, for his review of this manuscript.

References

1. Parkin DM, Bray F, Ferlay J, Pisani P. Estimating the world cancer burden: Globocan 2000. *Int J Cancer* 2001;**94**(2):153–6.
2. Matsuda T, Marugame T, Kamo K, et al. Japan Cancer Surveillance Research. Cancer incidence and incidence rates in Japan in 2003: based on data from 13 population-based cancer registries in the Monitoring of Cancer Incidence in Japan (MCIJ) project. *Jpn J Clin Oncol* 2009;**39**(12):850–8.
3. Tsukuma H, Ajiki W, Ioka A, et al. Research Group of Population-Based Cancer Registries of Japan. Survival of cancer patients diagnosed between 1993 and 1996: a collaborative study of population-based cancer registries in Japan. *Jpn J Clin Oncol* 2006;**36**(9):602–7.
4. Inoue K, Nakane Y, Michiura T, et al. Trends in long-term survival following surgery for gastric cancer: a single institution experience. *Oncol Rep* 2004;**11**(2):459–64.
5. Sakata Y, Ohtu A, Horikoshi N, Sugimachi K, Mitachi Y, Taguchi T. Late phase II study of novel oral fluoropyrimidine anticancer drug S-1 (1M tegaful-0.4M gimestat- 1M otastat potassium) in advanced gastric cancer patients. *Eur. J Cancer* 1998;**34**(11):1715–20.
6. Sugimachi K, Maehara Y, Horikoshi N, et al. An early phase II study of oral S-1, a newly developed 5-fluorouracil derivative for advanced and recurrent gastrointestinal cancers. The S-1 Gastrointestinal Cancer Study Group. *Oncology* 1999;**57**(3):202–10.
7. Koizumi W, Tanabe S, Saigenji K, et al. Phase I/II study of S-1 combined with cisplatin in patients with advanced gastric cancer. *Br J Cancer* 2003;**89**(12):2207–12.
8. Association, Japanese Gastric Cancer. Japanese classification of gastric carcinoma – 2nd English Edition. *Gastric Cancer* 1998;**1**(1): 10–24.
9. Therasse P, Arbuck SG, Eisenhauer EA, et al. New guidelines to evaluate the response to treatment in solid tumors. European Organization for Research and Treatment of Cancer, National Cancer Institute of the United States, National Cancer Institute of Canada. *J Natl Cancer Inst* 2000;**92**(3):205–16.
10. Takahashi S, Kinoshita T, Konishi M, et al. Phase II study of sequential high-dose methotrexate and fluorouracil combined with doxorubicin as a neoadjuvant chemotherapy for scirrhous gastric cancer. *Gastric Cancer* 2001;**4**:192–7.
11. Koizumi W, Narahara H, Hara T, et al. S-1 plus cisplatin versus S-1 alone for first-line treatment of advanced gastric cancer (SPIRITS trial): a phase III trial. *Lancet Oncol* 2008;**9**(3):215–21.
12. Yoshikawa T, Omura K, Kobayashi O, et al. A phase II study of preoperative chemotherapy with S-1 plus cisplatin followed by D2/D3 gastrectomy for clinically serosa-positive gastric cancer (JACCRO GC-01 study). *Eur J Surg Oncol* 2010;**36**(6):546–51.
13. Nakata B, Tsuji A, Mitachi Y, et al. Phase II trial of S-1 plus low-dose cisplatin for unresectable and recurrent gastric cancer (JFMC27-9902 Step2). *Oncology* 2010;**79**(5–6):337–42.

14. Yoshikawa T, Tsuburaya A, Morita S, et al. A comparison of multimodality treatment: two or four courses of paclitaxel plus cisplatin or S-1 plus cisplatin followed by surgery for locally advanced gastric cancer, a randomized Phase II trial (COMPASS). *Jpn J Clin Oncol* 2010; **40**(4):369–72.
15. Japan Clinical Oncology Group. Randomized phase III trial of surgery plus neoadjuvant TS-1 and cisplatin compared with surgery alone for type 4 and large type 3 gastric cancer: Japan Clinical Oncology Group Study (JCOG 0501). Clinical Trials. gov NCT00252161. http://clinicaltrials.gov/show/NCT00252161.
16. Cunningham D, Allum WH, Stenning SP, et al. MAGIC Trial Participants. Perioperative chemotherapy versus surgery alone for resectable gastroesophageal cancer. *N Engl J Med* 2006;**335**(1):11–20.
17. Sakuramoto S, Sasako M, Yamaguchi T, et al. Adjuvant chemotherapy for gastric cancer with S-1, an oral fluoropyrimidine. *N Engl J Med* 2007;**357**(18):1810–20.
18. Takahari D, Hamaguchi T, Yoshimura K, et al. Feasibility study of adjuvant chemotherapy with S-1 plus cisplatin for gastric cancer. *Cancer Chemother Pharmacol* 2010;**67**(6):1423–8.
19. Iwase H, Shimada M, Tsuzuki T, et al. A phase II multi-center study of triple therapy with paclitaxel, S-1 and cisplatin in patients with advanced gastric cancer. *Oncology* 2011;**80**(1–2):76–83.
20. Sato Y, Takayama T, Sagawa T, et al. Phase II study of S-1, docetaxel and cisplatin combination chemotherapy in patients with unresectable metastatic gastric cancer. *Cancer Chemother Pharmacol* 2010;**66**(4): 721–8.
21. Narahara H, Iishi H, Imamura H, et al. Randomized phase III study comparing the efficacy and safety of irinotecan plus S-1 with S-1 alone as firstline treatment for advanced gastric cancer (study GC0301/TOP-002). *Gastric Cancer* 2011;**14**(1):72–80.
22. Bang YJ, Van Cutsem E, Feyereislova A, et al. Trastuzumab in combination with chemotherapy versus chemotherapy alone for treatment of HER2-positive advanced gastric or gastro-oesophageal junction cancer (ToGA): a phase 3, open-label, randomised controlled trial. *Lancet* 2010;**376**(9742):687–97.
23. Sasako M, Sano T, Yamamoto S, et al. D2 lymphadenectomy alone or with para-aortic nodal dissection for gastric cancer. *N Engl J Med* 2008;**359**(5):453–62.
24. Yonemura Y, Wu CC, Fukushima N, et al. Randomized clinical trial of D2 and extended paraaortic lymphadenectomy in patients with gastric cancer. *Int J Clin Oncol* 2008;**13**(2):132–7.
25. Songun I, Putter H, Kranenbarg EM, Sasako M, van de Velde CJ. Surgical treatment of gastric cancer: 15-year follow-up results of the randomized nationwide Dutch D1D2 trial. *Lancet Oncol* 2010;**11**(5):439–49.
26. Hartgrink HH, van de Velde CJ, Putter H, et al. Extended lymph node dissection for gastric cancer: who may benefit? Final results of the randomized Dutch gastric cancer group trial. *J Clin Oncol* 2004;**22**(11): 2069–77.
27. Sano T, Sasako M, Yamamoto S, et al. Gastric cancer surgery: morbidity and mortality results from a prospective randomized controlled trial comparing D2 and extended para-aortic lymphadenectomy – Japan Clinical Oncology Group study 9501. *J Clin Oncol* 2004;**22**(14):2767–73.

Original Paper

European Surgical Research

Eur Surg Res 2011;47:274–283
DOI: 10.1159/000333833

Received: July 13, 2011
Accepted after revision: September 20, 2011
Published online: November 10, 2011

Active Hexose Correlated Compound Inhibits the Expression of Proinflammatory Biomarker iNOS in Hepatocytes

K. Matsui[a] T. Ozaki[a] M. Oishi[a] Y. Tanaka[a] M. Kaibori[a] M. Nishizawa[b] T. Okumura[a, c] A.-H. Kwon[a]

[a]Department of Surgery, Kansai Medical University, Moriguchi, [b]Department of Biomedical Sciences, College of Life Sciences, and [c]Research Organization of Science and Technology, Ritsumeikan University, Kusatsu, Japan

Key Words

Active hexose correlated compound · Interleukin-1β · iNOS · Nuclear factor-κB · Type I interleukin-1 receptor · iNOS gene antisense transcript

Abstract

Background/Aims: Excess production of nitric oxide (NO) by inducible nitric oxide synthase (iNOS) has been implicated as proinflammatory biomarker in liver injury. The application of active hexose correlated compound (AHCC) as a functional food in complementary and alternative medicine has increased. The possibility that AHCC might inhibit iNOS induction was investigated as a potential liver-protective effect. ***Methods:*** Hepatocytes were isolated from rats by collagenase perfusion and cultured. Primary cultured hepatocytes were treated with interleukin-1β in the presence or absence of AHCC-sugar fraction (AHCC-SF). ***Results and Conclusion:*** AHCC-SF inhibited the production of NO and reduced expressions of iNOS mRNA and its protein. AHCC-SF had no effects on either IκB degradation or nuclear factor-κB (NF-κB) activation. In contrast, AHCC-SF inhibited the upregulation of type I interleukin-1 receptor (IL-1RI) through the inhibition of Akt phosphorylation. Transfection experiments with iNOS promoter-luciferase constructs revealed that AHCC-SF reduced the levels of iNOS mRNA at both promoter transactivation and mRNA stabilization steps. AHCC-SF inhibited the expression of iNOS gene antisense transcript, which is involved in iNOS mRNA stabilization. These findings demonstrate that AHCC-SF suppresses iNOS gene expression through a IκB/NF-κB-independent but Akt/IL-1RI-dependent pathway, resulting in the reduction of NO production. AHCC-SF may have therapeutic potential for various liver injuries.

Introduction

In the liver, nitric oxide (NO) is produced by constitutively expressed endothelial nitric oxide synthase (eNOS) or inducible NOS (iNOS). eNOS is located in vascular sinusoidal endothelial cells, and NO produced by eNOS maintains hepatic circulation and endothelial integrity. iNOS is negligible under physiological conditions, but is expressed in hepatic cells including hepatocytes and Kupffer cells under pathological conditions such as sepsis, hemorrhagic shock, ischemia-reperfusion, hepatitis, and cirrhosis. During infection and inflammation in the liver, excess production of NO by iNOS is thought to be

KARGER

Fax +41 61 306 12 34
E-Mail karger@karger.ch
www.karger.com

0014–312X/11/0474–0274$38.00/0

Accessible online at:
www.karger.com/esr

Tadayoshi Okumura, PhD
Department of Surgery, Kansai Medical University
10–15 Fumizonocho
Moriguchi, Osaka 570-8506 (Japan)
Tel. +81 6 6993 9474, E-Mail okumura@takii.kmu.ac.jp

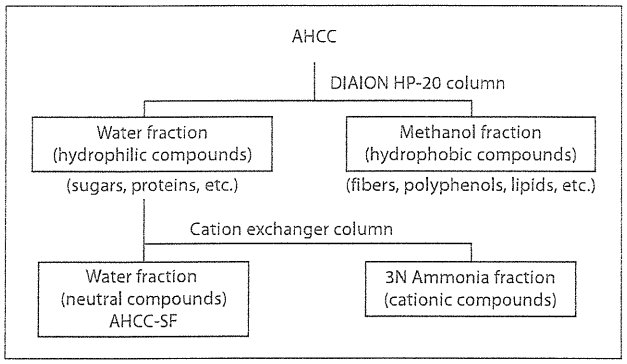

Fig. 1. Preparation of AHCC-SF. Separation flow of AHCC.

involved in liver injury. The expression of iNOS is a biomarker in proinflammation, although NO has been reported to exert either detrimental or beneficial effects depending on the insults and tissues involved. In our previous reports, clinical drugs, which showed liver-protective effects in various animal models of liver injury [1–5], prevented iNOS induction in the liver as well as decreased production of various inflammatory mediators. These drugs also inhibited iNOS induction and NO production in proinflammatory cytokine-stimulated cultured hepatocytes of rats [3, 6, 7], which is used as a simple in vitro injury model.

Proinflammatory cytokine interleukin (IL)-1β, or a mixture of IL-1β, tumor necrosis factor (TNF)-α and interferon-γ, induces the expression of iNOS gene in primary cultures of human and rat hepatocytes [8, 9]. The induction of iNOS is regulated by transactivation of the iNOS promoter with transcription factors including nuclear factor (NF)-κB, and by post-transcriptional modifications including mRNA stabilization [10]. There are two essential pathways involved in iNOS induction, IκB kinase/IκB/NF-κB activation and phosphatidylinositol-3 kinase (PI3K)/Akt/type I IL-1 receptor (IL-1RI) upregulation [11]. IL-1β stimulates the degradation of IκB after its phosphorylation by IκB kinase, which is followed by the translocation of NF-κB from cytoplasm to the nucleus and DNA binding (NF-κB activation). IL-1β also stimulates the upregulation of IL-1RI through activation of PI3K/Akt, which is essential for both transcriptional activation and mRNA stabilization in iNOS induction [7, 11–13]. In the case of mRNA stabilization, we have reported that natural iNOS gene antisense transcript interacts with 3′-untranslated region (UTR) containing AU-rich elements (ARE) of iNOS mRNA, leading to iNOS mRNA stabilization in IL-1β-stimulated hepatocytes [14].

The functional food active hexose correlated compound (AHCC) is an extract prepared from cultured mycelium of *Basidiomycetes* mushrooms. In recent reports [15–20], supplementation with AHCC has shown a generalized positive effect on the immune systems, as well as anti-inflammatory and anti-oxidant effects. AHCC is a mixture of polysaccharides, amino acids, lipids, and minerals, in which oligosaccharides are the major components constituting about 74% of the mixture. These oligosaccharides are believed to account for the biological activities of AHCC [21, 22]. In the liver, we reported that AHCC improved the prognosis of postoperative hepatocellular carcinoma patients [23]. However, the molecular mechanism by which AHCC protects the liver is not fully understood. In the current study, the possibility that AHCC might inhibit NO production was pursued as a possible liver-protecting mechanism. We intended to examine whether AHCC influences the induction of iNOS gene expression in primary cultures of rat hepatocytes, and if so, study the mechanism involved in this process.

Materials and Methods

Materials

Recombinant human IL-1β (2×10^7 U/mg protein) was provided by Otsuka Pharmaceutical Co. Ltd. (Tokushima, Japan). [γ-^{32}P]adenosine-5′-triphosphate (ATP; –222 TBq/mmol) and [α-^{32}P]deoxycytidine-5′-triphosphate (dCTP; –111 TBq/mmol) were obtained from DuPont-New England Nuclear Japan (Tokyo, Japan). Rats were kept at 22°C under a 12-h light/12-h dark cycle, and received food and water at libitum. All animal experiments were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals of the National Institutes of Health, and approved by the Animal Care Committee of Kansai Medical University.

Preparation of Sugar Fraction of AHCC

As shown in figure 1, the extract of AHCC (20 g), which was supplied by Amino Up Chemical Co. Ltd (Sapporo, Japan), was dissolved in H_2O (80 ml), applied on the column of DIAION HP-20 (5 × 25 cm; Mitsubishi Chemical Co., Japan) and eluated with H_2O (1 liter) and methanol. The first eluate (water fraction containing hydrophilic compounds) was concentrated under vacuum, followed by lyophilization (18.1 g of yellowish powder). The water fraction was dissolved in H_2O (35 ml), mixed with methanol (180 ml) and centrifuged (1,600 *g* for 15 min), which was repeated twice. Then the precipitate was dissolved in H_2O (100 ml), applied on the DOWEX 50 WX8 (4.4 × 17 cm; The Dow Chemical Company, USA), and eluted with H_2O (0.5 liter) and ammonia (3 N). The final water fraction containing AHCC-sugar fraction (AHCC-SF) was lyophilized (4.93 g) and stored at –20°C.

Primary Cultures of Hepatocytes

Hepatocytes were isolated from male Wistar strain rats (200–220 g; Charles River, Tokyo, Japan) by collagenase (Wako Pure Chemicals, Osaka, Japan) perfusion [24, 25]. Isolated hepatocytes were suspended in culture medium at 6×10^5 cells/ml, seeded into 35-mm plastic dishes (2 ml/dish; Falcon Plastic, Oxnard, Calif., USA) and cultured at 37°C in a CO_2 incubator under a humidified atmosphere of 5% CO_2 in air. The culture medium was Williams' medium E (WE) supplemented with 10% newborn calf serum, HEPES (5 mM), penicillin (100 U/ml), streptomycin (0.1 mg/ml), dexamethasone (10 nM) and insulin (10 nM). After 5 h, the medium was replaced with fresh serum- and hormone-free WE, and the cells were cultured overnight before use in experiments. The numbers of cells attached to the dishes were calculated by counting the nuclei [26] and using a ratio of 1.37 ± 0.04 nuclei/cell (mean ± SE, n = 7 experiments).

Treatment of Cells with AHCC-SF

On day 1, the cells were washed with fresh serum- and hormone-free WE, and incubated with IL-1β (1 nM) in the same medium in the presence or absence of AHCC-SF.

Determinations of NO Production

Culture media were used for measurements of nitrite (stable metabolites of NO) for NO production by the Griess method [27].

Western Blot Analysis

Total cell lysates were obtained from cultured cells [6], mixed with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (final concentrations: 125 mM Tris-HCl, pH 6.8, 5% glycerol, 2% SDS and 1% 2-mercaptoethanol), subjected to SDS-PAGE and transferred onto polyvinylidene difluoride membranes (Bio-Rad Laboratories, Hercules, Calif., USA). Immunostaining was performed using primary antibodies against mouse iNOS (Affinity BioReagents, Golden, Colo., USA), human IκBα, human IκBβ, mouse IL-1RI (Santa Cruz Biotechnology, Santa Cruz, Calif., USA) and rat β-tubulin (internal control; Clone TUB2.1; Sigma Chemical Co., St. Louis, Mo., USA), followed by visualization with an ECL blotting detection reagent (GE Healthcare Biosciences Corp., Piscataway, N.J., USA).

In the case of Akt, total cell lysates prepared from 100-mm dishes (5×10^6 cells/dish) were precleared with Protein A (Sigma Chemical Co.), and then mixed with a mouse monoclonal antibody against human Akt1 (Akt5G3; Cell Signaling, Beverly, Mass., USA) and Protein G-Sepharose (Pharmacia LKB Biotech, Uppsala, Sweden). After incubation overnight at 4°C, the immunocomplexes were centrifuged (16,000 *g* for 5 min). The beads were washed with solubilizing buffer, dissolved in SDS-PAGE sample buffer, and analyzed by western blotting using rabbit polyclonal antibodies against human Akt and phospho-(Ser473) Akt (Cell Signaling) as primary antibodies.

Northern Blot Analysis and RT-PCR

Total RNA was extracted from cultured hepatocytes using a guanidinium-phenol-chloroform method [28] with Trizol reagent (Invitrogen, Carlsbad, Calif., USA) according to the manufacturer's instructions. Next, 10 μg of total RNA was fractionated by 1% agarose-formaldehyde gel electrophoresis, transferred to nylon membranes (Nytran; Schleicher and Schuell, Dassel, Germany), immobilized by baking at 80°C for 1 h and hybridized with DNA probes. A cDNA probe for rat iNOS (830 bp) was described previously [29]. cDNAs encoding rat IL-1RI [30] and mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [31] were prepared by RT-PCR [32]. The cDNAs were radiolabeled with [α-^{32}P]dCTP by the random priming method.

For strand-specific RT-PCR analysis, cDNAs were synthesized from total RNA with strand-specific primers, and step-down PCR was performed as previously described [32, 33]. For iNOS and elongation factor (EF)-1α (internal control) mRNAs, an oligo(dT) primer was used for RT and primer sets 5′-CCAACCTGCAGGTCTTCGATG-3′ and 5′-GTCGATGCACAACTGGGTGAAC-3′ (257-bp product) and 5′-TCTGGTTGGAATGGTGACAACATGC-3′ and 5′-CCAGGAAGAGCTTCACTCAAAGCTT-3′ (307-bp product) were used for PCR, respectively. For the antisense transcript of iNOS gene, sense primer 5′-TGCCCCTCCCCCACATTCTCT-3′ was used for RT and the primer set 5′-ACCAGGAGGCGCCATCCCGCTGC-3′ and 5′-CTTGATCAAACACTCATTTTATTAAA-3′ (186-bp product) were used for PCR. The iNOS mRNA and antisense transcript levels were measured in triplicate by real-time PCR using an iCycler System (Bio-Rad Laboratories). SYBR Green I (Roche Diagnostics) was included in the reaction mixture, and the following touchdown protocol was applied: 1 cycle of 94°C for 1 min; and 50 cycles of 94°C for 30 s, (72–0.3 × n)°C for 1 min where n is the number of cycles, and 72°C for 30 s. Rat cDNAs for the iNOS mRNA and antisense transcript were deposited in DDBJ/EMBL/GenBank under accession No. AB250951 and AB250952, respectively.

Electrophoretic Mobility Shift Assay (EMSA)

Nuclear extracts were prepared according to Schreiber et al. [34] with minor modifications [35]. Briefly, the dishes were placed on ice, washed with Tris-HCl-buffered saline, harvested with the same buffer using a rubber policeman and centrifuged (1,840 *g* for 1 min). The precipitate (2×10^6 cells from two 35-mm dishes) was suspended in 400 μl of lysis buffer (10 mM Hepes, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 500 U/ml trasylol, 0.5 mM PMSF and 1 mM dithiothreitol) and incubated on ice for 15 min. After addition of Nonidet P-40 (final: 0.625%), the cells were lysed by vortexing (2–3 times for 1 min each) and centrifuged (15,000 *g* for 1 min). The nuclear pellet was resuspended with extraction buffer (10 mM Hepes, pH 7.9, 0.4 M NaCl, 0.1 mM EDTA, 0.1 mM EGTA, 500 U/ml trasylol, 0.5 mM PMSF and 1 mM dithiothreitol), followed by continuous mixing for 20 min and centrifugation (15,000 *g* for 5 min). Aliquots of the supernatant (nuclear extract) were frozen in liquid nitrogen and stored at –80°C until use.

Binding reactions (total: 15 μl) were performed by incubating nuclear extract aliquots (4 μg of protein) in reaction buffer (20 mM Hepes, pH 7.9, 1 mM EDTA, 60 mM KCl, 10% glycerol and 1 mg of poly(dI-dC)) with the probe (approximately 40,000 c.p.m.) for 20 min at room temperature. In the case of supershift assays, the nuclear extracts were incubated in the presence of anti-p50 and anti-p65 antibodies (NF-κBp50 (NLS) and NF-κBp65 (H286); Santa Cruz Biotechnology) or cold probes as a competitor (250-fold excess) for 30 min at 4°C, followed by incubation with the labeled probe. The products were electrophoresed at 100 V in a 4.8% polyacrylamide gel in high ionic strength buffer (50 mM Tris-HCl, 380 mM glycine, 2 mM EDTA, pH 8.5) and the dried gels were analyzed by autoradiography. An NF-κB consensus oligonucleotide (5′-AGTTGAGGGGA-CTTTCCCAGGC-3′) from the mouse immunoglobulin κ light chain was purchased (Promega,

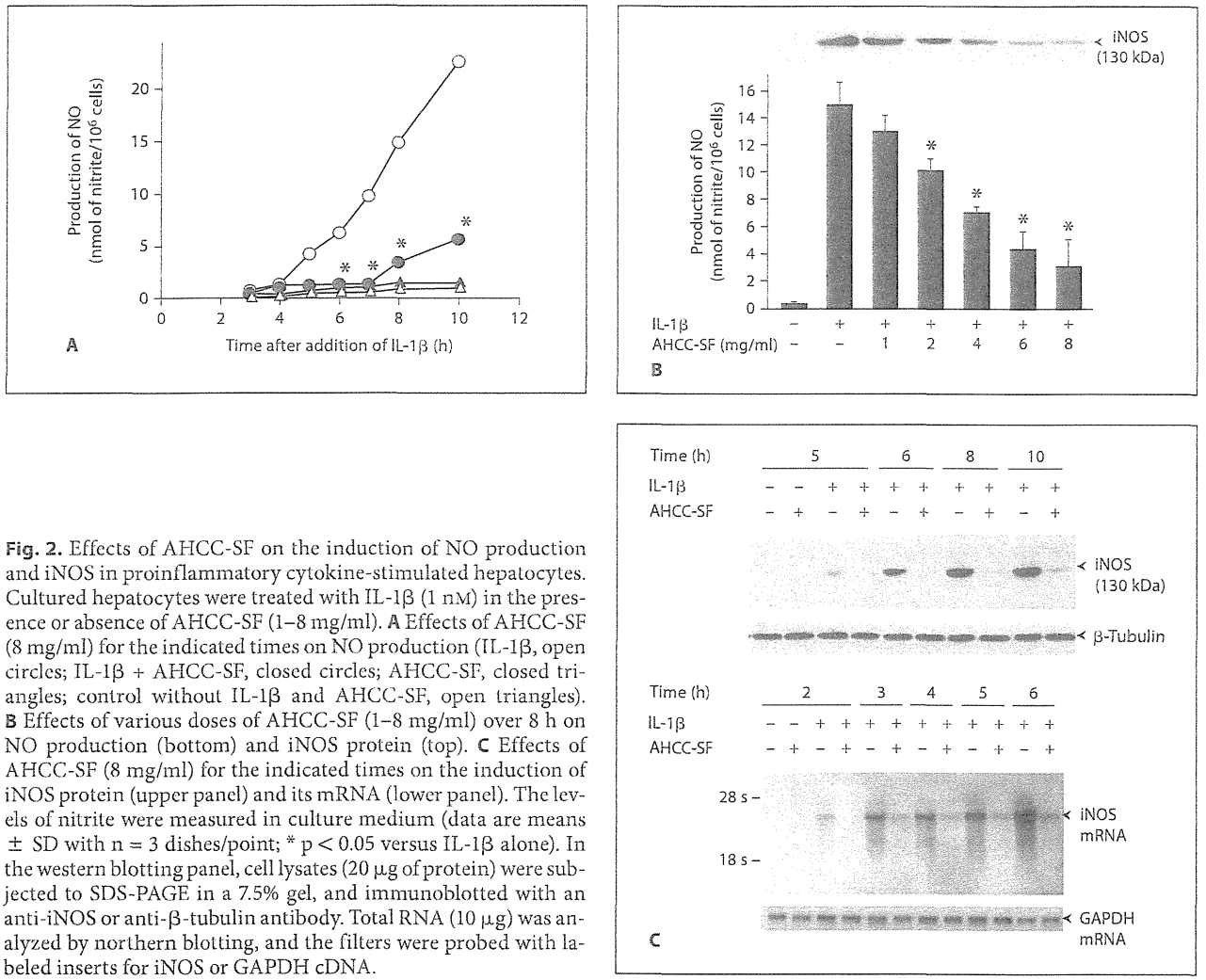

Fig. 2. Effects of AHCC-SF on the induction of NO production and iNOS in proinflammatory cytokine-stimulated hepatocytes. Cultured hepatocytes were treated with IL-1β (1 nM) in the presence or absence of AHCC-SF (1–8 mg/ml). **A** Effects of AHCC-SF (8 mg/ml) for the indicated times on NO production (IL-1β, open circles; IL-1β + AHCC-SF, closed circles; AHCC-SF, closed triangles; control without IL-1β and AHCC-SF, open triangles). **B** Effects of various doses of AHCC-SF (1–8 mg/ml) over 8 h on NO production (bottom) and iNOS protein (top). **C** Effects of AHCC-SF (8 mg/ml) for the indicated times on the induction of iNOS protein (upper panel) and its mRNA (lower panel). The levels of nitrite were measured in culture medium (data are means ± SD with n = 3 dishes/point; * $p < 0.05$ versus IL-1β alone). In the western blotting panel, cell lysates (20 μg of protein) were subjected to SDS-PAGE in a 7.5% gel, and immunoblotted with an anti-iNOS or anti-β-tubulin antibody. Total RNA (10 μg) was analyzed by northern blotting, and the filters were probed with labeled inserts for iNOS or GAPDH cDNA.

Madison, Wisc., USA) and labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase. The protein concentration was measured by the method of Bradford [36] with a binding assay kit (Bio-Rad Laboratories) using bovine serum albumin as a standard.

Construction of Luciferase Reporter Plasmids and Expression Plasmids

The 1.2-kb 5′-flanking region including the TATA box of the rat iNOS gene was inserted into the pGL3-Basic vector (Promega) to create pRiNOS-Luc-SVpA [35]. A rat cDNA for the 3′-UTR of the iNOS mRNA was amplified with the primers 5′-tgctctaGACAGTGAGGGGTTTGGAGAGA-3′ and 5′-gcggatccttta-TTCTTGATCAAACACTCATTTT-3′, and the resultant cDNA was digested with BamH I and Xba I. This cDNA for the iNOS 3′-UTR (submitted to DDBJ/EMBL/GenBank under accession No. AB250951) was used to replace the SV40 polyadenylation signal (SVpA) of pRiNOS-Luc to create pRiNOS-Luc-3′-UTR [12].

Transfection and Luciferase Assay

Transfection of cultured hepatocytes was performed as described previously [37, 38]. Briefly, hepatocytes were cultured at 4×10^5 cells/dish (35 × 10 mm) in WE supplemented with serum, dexamethasone and insulin for 7 h, before being subjected to magnet-assisted transfection (MATra). Reporter plasmids pRiNOS-Luc-SVpA or pRiNOS-Luc-3′-UTR (1 μg) and the CMV promoter-driven β-galactosidase plasmid pCMV-LacZ (1 ng) as an internal control were mixed with MATra-A reagent (1 μl; IBA GmbH, Göttingen, Germany). After incubation for 15 min on a magnetic plate at room temperature, the medium was replaced with fresh WE containing serum. The cells were cultured overnight, and then treated with IL-1β in the presence or absence of AHCC-SF. The luciferase and β-galactosidase activities of cell extracts were measured using PicaGene (Wako Pure Chemicals) and Beta-Glo (Promega) kits, respectively.

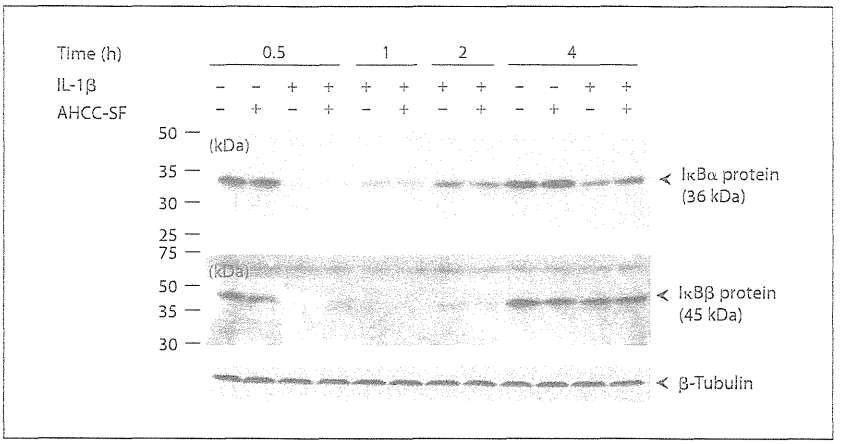

Fig. 3. Effects of AHCC-SF on the degradation of IκB proteins. Cells were treated with IL-1β (1 nM) in the presence or absence of AHCC-SF (8 mg/ml) for the indicated times. Cell lysates (20 μg of protein) were subjected to SDS-PAGE in a 12.5% gel, followed by immunoblotting with an anti-IκBα, anti-IκBβ or anti-β-tubulin antibody.

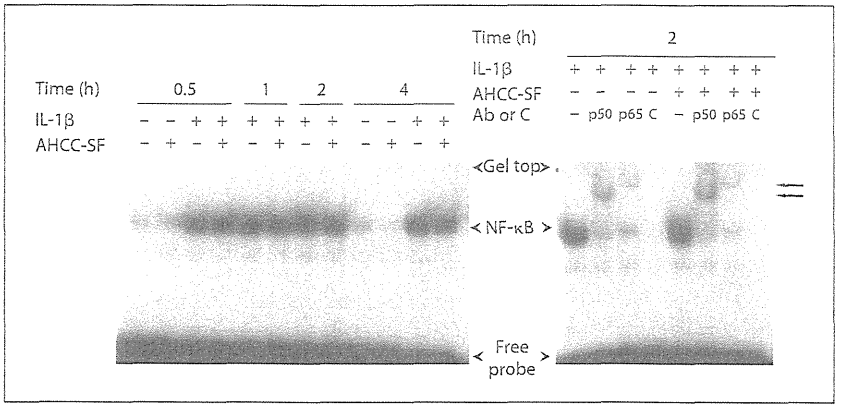

Fig. 4. Effects of AHCC-SF on the activation of NF-κB. Cells were treated with IL-1β (1 nM) in the presence or absence of AHCC-SF (8 mg/ml) for the indicated times. Activation of NF-κB (left): nuclear extracts (4 μg of protein) were analyzed by EMSAs. Supershift assay (right): nuclear extracts were incubated with a labeled NF-κB consensus oligonucleotide in the presence of an anti-p50 antibody, anti-p65 antibody or cold probe as a competitor (C, 250-fold excess). Closed arrows show supershifted bands.

Statistical Analysis

The results shown in the figures are representative of 3–4 independent experiments yielding similar findings. Differences were analyzed by the Bonferroni-Dunn test, and values of $p < 0.05$ were considered to indicate statistical significance.

Results

AHCC-SF Inhibits iNOS Induction in IL-1β-Stimulated Hepatocytes

The proinflammatory cytokine IL-1β stimulates the induction of iNOS gene expression in primary cultures of rat hepatocytes [9, 37]. Simultaneous addition of AHCC-SF (1–8 mg/ml) with IL-1β decreased the production of NO time- and dose-dependently (fig. 2A, B). AHCC-SF had a maximal effect (over 80% inhibition) at 8 mg/ml, but showed no cellular cytotoxicity as evaluated by release of lactate dehydrogenase into the culture medium and Trypan blue exclusion in hepatocytes (data not shown). Western and northern blot analyses revealed that AHCC-SF decreased the levels of iNOS protein (fig. 2B, C, upper panel) and iNOS mRNA (fig. 2C, lower panel), suggesting that it inhibited the induction of iNOS gene expression at a transcriptional and/or post-transcriptional step.

AHCC-SF Has No Effects on IκB Degradation and NF-κB Activation

We examined the mechanism involved in the inhibition of iNOS induction. AHCC-SF did not influence the degradation of IκBα and IκBβ proteins at 0.5 h and their recovery at 1–4 h (fig. 3). EMSAs with the nucleus revealed that AHCC-SF had no effect on the activation of NF-κB (fig. 4, left). Furthermore, supershift experiments showed that AHCC-SF also had no effect on the components of NF-κB subunits (p50 and p65) (fig. 4, right), since the NF-κB bands stimulated by IL-1β disappeared similarly in the presence of antibodies against p50 and p65, irrespective of the presence of AHCC-SF.

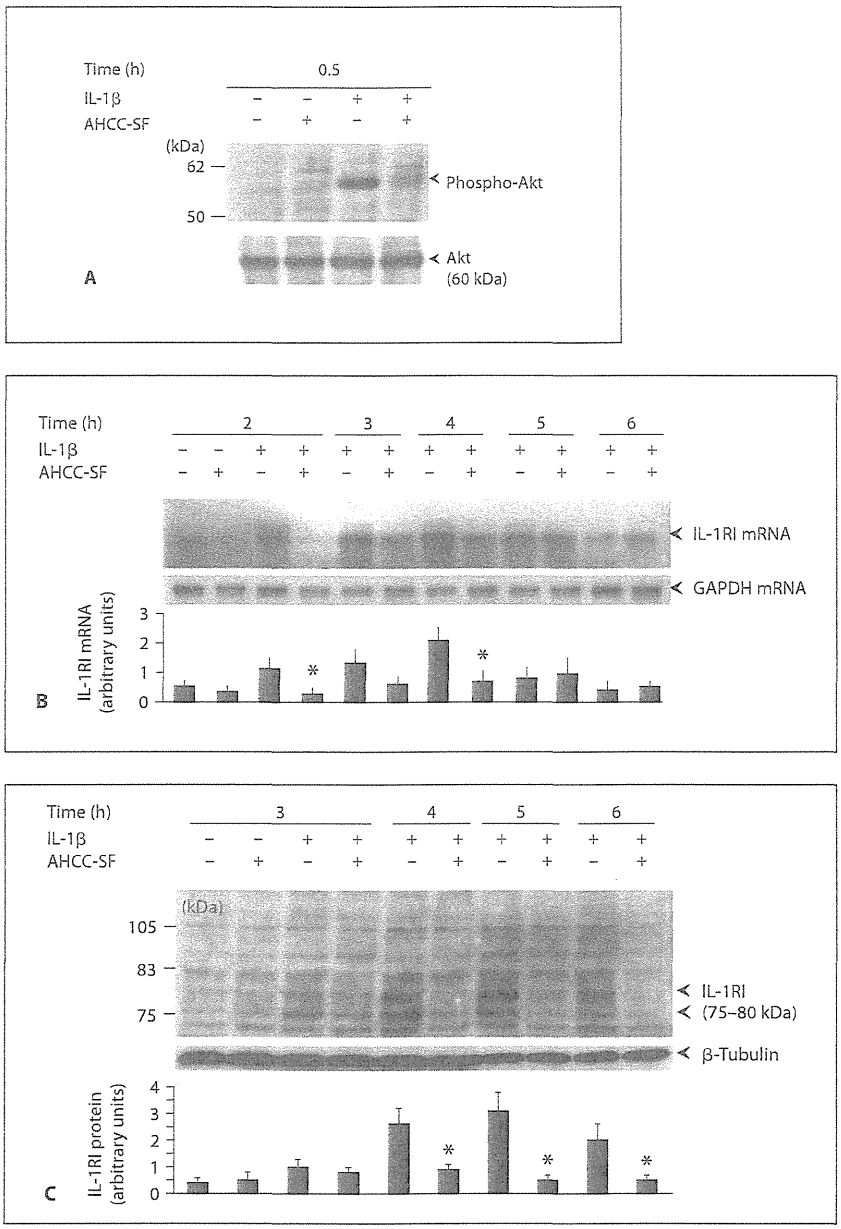

Fig. 5. Effects of AHCC-SF on the upregulation of IL-1RI. Cells were treated with IL-1β (1 nM) in the presence or absence of AHCC-SF (8 mg/ml) for the indicated times. **A** Activation of Akt: total cell lysates were immunoprecipitated with an anti-Akt antibody, followed by immunoblotting with an anti-phospho-Akt or anti-Akt antibody. **B** Total RNA (10 μg) was analyzed by northern blotting, and the filters were probed with labeled inserts for IL-1RI or GAPDH cDNA. **C** Cell lysates (50 μg of protein) were subjected to SDS-PAGE in a 7.5% gel, and immunoblotted with an anti-IL-1RI or anti-β-tubulin antibody. The bands corresponding to IL-1RI mRNA or protein were quantified by densitometry (lower panels: means ± SD for n = 3 experiments; * $p < 0.05$ vs. IL-1β alone).

AHCC-SF Decreases Upregulation of IL-1RI

IL-1β stimulates the upregulation of IL-1RI through activation of PI3K/Akt [11], which is essential for the induction of iNOS gene expression in addition to NF-κB activation in hepatocytes. AHCC-SF reduced the phosphorylation of Akt (fig. 5A), which is a downstream kinase of PI3K. AHCC-SF also inhibited the increased expressions of IL-1RI mRNA and its protein (fig. 5B, C). These observations suggest that AHCC-SF can influence the downstream events of IL-1RI upregulation, but not through IκB degradation and NF-κB activation.

AHCC-SF Affects iNOS mRNA Synthesis and Stabilization

Next, we carried out transfection experiments with iNOS promoter-firefly luciferase constructs, namely

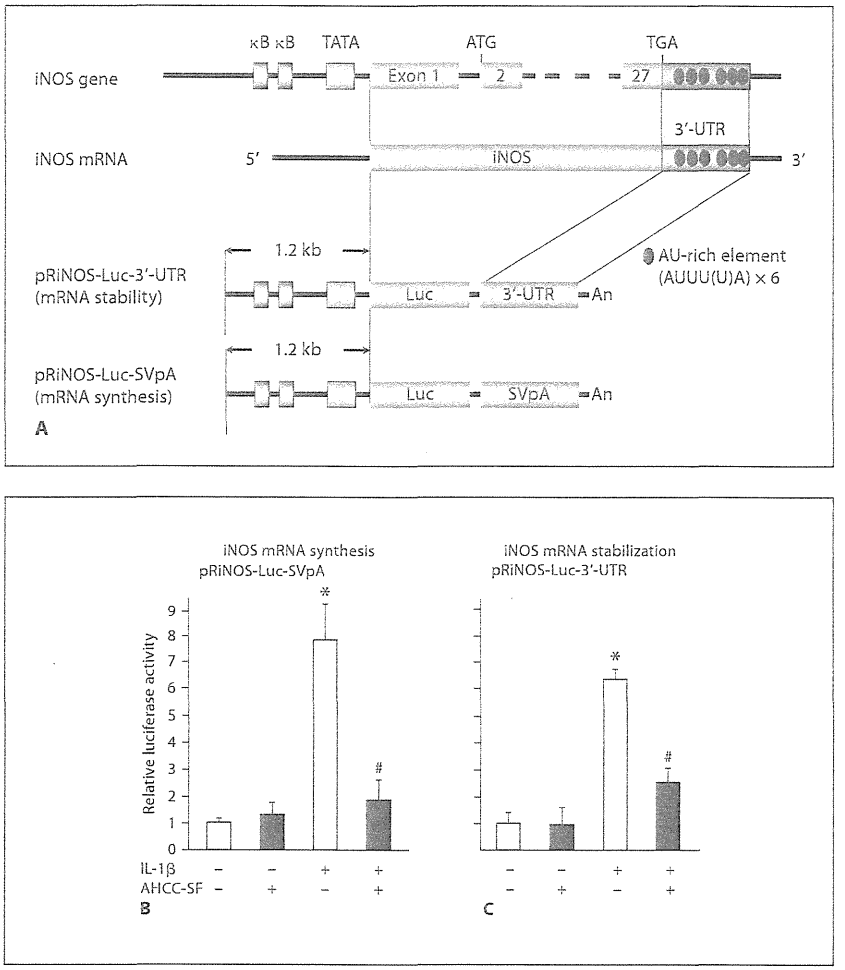

Fig. 6. Effects of AHCC-SF on the transactivation of the iNOS promoter. **A** Schematic representation of the promoter region of the iNOS gene. Two reporter constructs are shown beneath the iNOS gene and mRNA. The constructs consist of the rat iNOS promoter (1.2 kb), luciferase gene and SV40 poly(A) region (pRiNOS-Luc-SVpA) or iNOS 3′-UTR (pRiNOS-Luc-3′-UTR). 'An' indicates the presence of a poly(A) tail. The iNOS 3′-UTR contains AREs (AUUU(U)A × 6), which contribute to mRNA stabilization. **B**, **C** Each construct was introduced into hepatocytes, and the cells were treated with IL-1β (1 nM) in the presence or absence of AHCC-SF (8 mg/ml) for 8 h for pRiNOS-Luc-SVpA (**B**) and 4 h for pRiNOS-Luc-3′UTR (**C**). The luciferase activities were normalized by the β-galactosidase activity. The fold activation was calculated by dividing the luciferase activity by that of the control (without IL-1β and AHCC-SF). Data are means ± SD (n = 4 dishes). * $p < 0.05$ versus control, # $p < 0.05$ versus IL-1β alone.

pRiNOS-Luc-SVpA and pRiNOS-Luc-3′-UTR (fig. 6A), which detect the activities of iNOS promoter transactivation (iNOS mRNA synthesis) and iNOS mRNA stabilization, respectively [12, 39]. IL-1β increased the luciferase activities of these constructs, and AHCC-SF significantly reduced both of these luciferase activities (fig. 6B, C). Recently, we found that the natural antisense transcript of the iNOS gene is expressed and involved in the stabilization of iNOS mRNA [14]. RT-PCR and quantitative real-time PCR experiments revealed that IL-1β time-dependently increased the expression of the iNOS gene antisense transcript (fig. 7A) with increased levels of iNOS mRNA (fig. 7B), and that AHCC-SF decreased the levels of both the antisense transcript and iNOS mRNA in a similar manner.

Discussion

In the present study, AHCC-SF was found to inhibit iNOS induction at the steps of both its promoter transactivation (mRNA synthesis) and mRNA stabilization in proinflammatory cytokine-stimulated hepatocytes (fig. 6). In the former, although AHCC-SF reduced the activities of iNOS promoter transactivation, AHCC-SF had no effects on IκB degradation (fig. 3) and NF-κB activation (fig. 4), indicating that AHCC-SF cannot influence the nuclear translocation of NF-κB and its DNA binding in IκB kinase signaling. In concert with IκB degradation/NF-κB activation, the upregulation of IL-1RI, which stimulates the phosphorylation of NF-κB subunit p65, is required for transcriptional activation of the iNOS gene, as we reported previously [11]. In the present study,

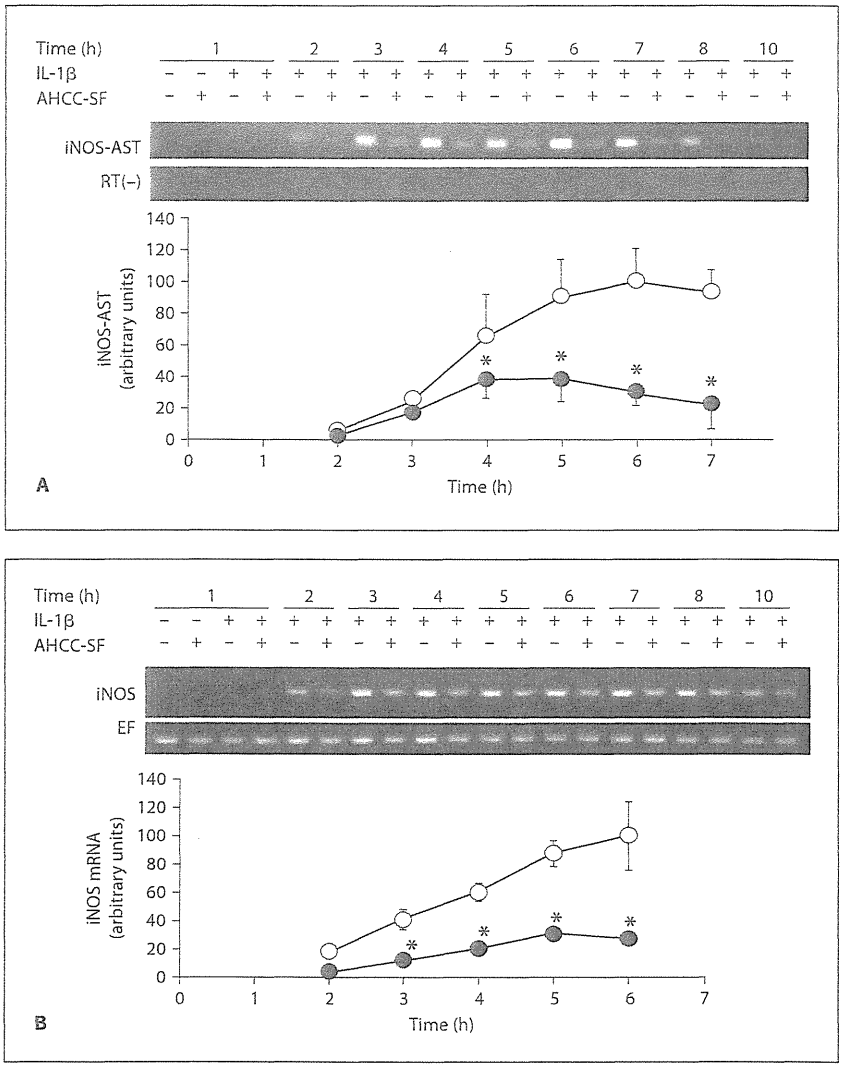

Fig. 7. Effects of AHCC-SF on the expression of the iNOS gene antisense transcript in hepatocytes. Cells were treated with IL-1β (1 nM) in the presence (closed circles) or absence (open circles) of AHCC-SF (8 mg/ml) for the indicated times. Total RNA was analyzed by strand-specific RT-PCR to detect (**A**) the iNOS gene antisense transcript (AST) and (**B**) iNOS with elongation factor-1α (EF) as an internal control. Quantitative RT-PCR was carried out for the iNOS gene AST or iNOS, and the copy number of the iNOS gene AST or iNOS was normalized by that of a negative PCR control using total RNA without RT (RT(–)) or by that of internal control EF. Data are means ± SD (n = 3 experiments). * $p < 0.05$ versus IL-1β alone.

we found that AHCC-SF decreased the expression of IL-1RI mRNA and protein (fig. 5B, C) through the inhibition of Akt phosphorylation (fig. 5A) in PI3K/Akt signaling, presumably leading to the inhibition of p65 phosphorylation and resulting in decreased activities of iNOS promoter transactivation (fig. 6B).

Regarding the iNOS mRNA stabilization, the 3′-UTR of the iNOS mRNA in rats has six AREs (AUUU(U)A), which are associated with ARE-binding proteins such as HuR and heterogeneous nuclear ribonucleoproteins L/I (PTB), thus contributing to the stabilization of the mRNA [40]. Recently, we found that the antisense strand corresponding to the 3′-UTR of iNOS mRNA is transcribed from the iNOS gene, and that the iNOS mRNA antisense transcript plays a key role in stabilizing the iNOS mRNA by interacting with the 3′-UTR and ARE-binding proteins [14]. In our in vitro model, AHCC-SF prevented the stabilization of iNOS mRNA (fig. 6C) by decreasing the iNOS gene antisense transcript expression (fig. 7A). Drugs such as edaravone (free radical scavenger) [7], FR183998 (Na^+/H^+ exchanger inhibitor) [3, 5], insulin-like growth factor I [4] and dexamethasone [41] were found to inhibit iNOS induction partly by suppressing iNOS antisense transcript production in primary cultured hepatocytes (our in vitro model) and in animal models of liver injury. In the case of dexamethasone, it

had no effects on either NF-κB activation or IL-1RI upregulation as compared with AHCC-SF. Dexamethasone inhibited the stabilization of iNOS mRNA but had no effect on the iNOS promoter transactivation [41], suggesting that IL-1RI upregulation as well as NF-κB activation is involved in transcriptional activation of the iNOS gene as mentioned before.

Our in vitro results suggest that AHCC-SF might inhibit the induction of iNOS expression and NO production in liver injury, which leads to liver-protective effects. Although such liver-protective effects deduced from our in vitro model need to be examined and supported in in vivo animal models of liver injury, our simple model with cultured hepatocytes may be adequate for the screening of liver-protective drugs, because it is rapid and inexpensive compared with animal models. In conclusion, AHCC-SF inhibited iNOS gene expression at transcriptional and post-transcriptional steps in cultured hepatocytes in an in vitro liver injury model. AHCC may have liver-protective effects for various liver injuries.

Acknowledgements

This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Culture and Sports of Japan, and by grants from the Science Research Promotion Fund of the Japan Private School Promotion Foundation.

References

1 Tsuchiya H, Kaibori M, Yanagida H, Yokoigawa N, Kwon AH, Okumura T, Kamiyama Y: Pirfenidone prevents endotoxin-induced liver injury after partial hepatectomy in rats. J Hepatol 2004;40:94–101.
2 Tsuji K, Kwon AH, Yoshida H, Qiu Z, Kaibori M, Okumura T, Kamiyama Y: Free radical scavenger (edaravone) prevents endotoxin-induced liver injury after partial hepatectomy in rats. J Hepatol 2005;42:94–101.
3 Tanaka T, Uchida Y, Kaibori M, Hijikawa T, Ishizaki M, Yamada M, Matsui K, Ozaki T, Tokuhara K, Kamiyama Y, Nishizawa M, Ito S, Okumura T: Na^+/H^+ exchanger inhibitor, FR183998, has protective effect in lethal acute liver failure and prevents iNOS induction in rats. J Hepatol 2008;48:289–299.
4 Hijikawa T, Kaibori M, Uchida Y, Yamada M, Matsui K, Ozaki T, Kamiyama Y, Nishizawa M, Okumura T: Insulin-like growth factor-I prevents liver injury through the inhibition of TNF-α and iNOS induction in D-galactosamine and lipopolysaccharide-treated rats. Shock 2008;29:740–747.
5 Ishizaki M, Kaibori M, Uchida Y, Hijikawa T, Tanaka H, Ozaki T, Tokuhara K, Matsui K, Kwon AH, Kamiyama Y, Nishizawa M, Okumura T: Protective effect of FR183998, a Na^+/H^+ exchanger inhibitor, and its inhibition of iNOS induction in hepatic ischemia-reperfusion injury in rats. Shock 2008;30:311–317.
6 Nakanishi H, Kaibori M, Teshima S, Yoshida H, Kwon AH, Kamiyama Y, Nishizawa M, Ito S, Okumura T: Pirfenidone inhibits the induction of iNOS stimulated by interleukin-1β at a step of NF-kappaB DNA binding in hepatocytes. J Hepatol 2004;41:730–736.
7 Yoshida H, Kwon AH, Kaibori M, Tsuji K, Habara K, Yamada M, Kamiyama Y, Nishizawa M, Ito S, Okumura T: Edaravone prevents iNOS expression by inhibiting its promoter transactivation and mRNA stability in cytokine-stimulated hepatocytes. Nitric Oxide 2008;18:105–112.
8 Geller DA, de Vera ME, Russell DA, Shapiro RA, Nussler AK, Simmons RL, Billiar TR: A central role for IL-1 beta in the in vitro and in vivo regulation of hepatic inducible nitric oxide synthase. IL-1 beta induces hepatic nitric oxide synthesis. J Immunol 1995;155:4890–4898.
9 Kitade H, Sakitani K, Inoue K, Masu Y, Kawada N, Hiramatsu Y, Kamiyama Y, Ito S, Okumura T: Interleukin-1β markedly stimulates nitric oxide formation in the absence of other cytokines or lipopolysaccharide, in primary cultured rat hepatocytes, but not in Kupffer cells. Hepatology 1996;23:797–802.
10 Kleinert H, Pautz A, Linker K, Schwarz PM: Regulation of the expression of inducible nitric oxide synthase. Eur J Pharmacol 2004;500:255–266.
11 Teshima S, Nakanishi H, Nishizawa M, Kitagawa K, Kaibori M, Yamada M, Habara K, Kwon AH, Kamiyama Y, Ito S, Okumura T: Up-regulation of IL-1 receptor through PI3K/Akt is essential for the induction of iNOS gene expression in hepatocytes. J Hepatol 2004;40:616–623.
12 Yamada M, Nishizawa M, Nakatake R, Habara K, Yoshida H, Ozaki T, Matsui K, Hamada Y, Kamiyama Y, Ito S, Okumura T: Characterization of alternatively spliced isoforms of the type I interleukin-1 receptor on iNOS induction in rat hepatocytes. Nitric Oxide 2007;17:98–105.
13 Habara K, Hamada Y, Yamada M, Tokuhara K, Tanaka H, Kaibori M, Kamiyama Y, Nishizawa M, Ito S, Okumura T: Pitavastatin up-regulates the induction of iNOS through enhanced stabilization of its mRNA in pro-inflammatory cytokine-stimulated hepatocytes. Nitric Oxide 2008;18:19–27.
14 Matsui K, Nishizawa M, Ozaki T, Kimura T, Hashimoto I, Yamada M, Kaibori M, Kamiyama Y, Ito S, Okumura T: Natural antisense transcript stabilizes inducible nitric oxide synthase mRNA in rat hepatocytes. Hepatology 2008;47:686–697.
15 Aviles H, O'Donnell P, Sun B, Sonnenfeld G: Active hexose correlated compound (AHCC) enhances resistance to infection in a mouse model of surgical wound infection. Surg Infect (Larchmt) 2006;7:527–535.
16 Ritz BW, Nogusa S, Ackerman EA, Gardner EM: Supplementation with active hexose correlated compound increases the innate immune response of young mice to primary influenza infection. J Nutr 2006;136:2868–2873.
17 Daddaoua A, Martínez-Plata E, López-Posadas R, Vieites JM, González M, Requena P, Zarzuelo A, Suárez MD, de Medina FS, Martínez-Augustin O: Active hexose correlated compound acts as a prebiotic and is antiinflammatory in rats with hapten-induced colitis. J Nutr 2007;137:1222–1228.
18 Fujii H, Nishioka H, Wakame K, Sun B: Nutritional food active hexose correlated compound (AHCC) enhances resistance against bird flu. Nihonhokandaigaeiryougakkaishi (Journal of Japanese Complementary and Alternative Medicine) 2007;4:37–40.
19 Aviles H, O'Donnell P, Orshal J, Fujii H, Sun B, Sonnenfeld G: Active hexose correlated compound activates immune function to decrease bacterial load in a murine model of intramuscular infection. Am J Surg 2008;195:537–545.
20 Terakawa N, Matsui Y, Satoi S, Yanagimoto H, Takahashi K, Yamamoto T, Yamao J, Takai S, Kwon AH, Kamiyama Y: Immunological effect of active hexose correlated compound (AHCC) in healthy volunteers: a double-blind, placebo-controlled trial. Nutr Cancer 2008;60:643–651.

21 Sakagami H, Aoki T: Induction of immunopotentiation activity by a protein-bound polysaccharide, PSK. Anticancer Res 1991; 11:993–1000.
22 Ye SF, Ichimura K, Wakame K, Ohe M: Suppressive effects of Active Hexose Correlated Compound on the increased activity of hepatic and renal ornithine decarboxylase induced by oxidative stress. Life Sci 2003;74: 593–602.
23 Matsui Y, Uhara J, Satoi S, Kaibori M, Yamada H, Kitade H, Imamura A, Takai S, Kawaguchi Y, Kwon AH, Kamiyama Y: Improved prognosis of postoperative hepatocellular carcinoma patients when treated with functional foods: a prospective cohort study. J Hepatol 2002;37:78–86.
24 Seglen PO: Preparation of isolated rat liver cells. Methods Cell Biol 1976;13:29–83.
25 Kanemaki T, Kitade H, Hiramatsu Y, Kamiyama Y, Okumura T: Stimulation of glycogen degradation by prostaglandin E_2 in primary cultured rat hepatocytes. Prostaglandins 1993;45:459–474.
26 Horiuti Y, Ogishima M, Yano K, Shibuya Y: Quantification of cell nuclei isolated from hepatocytes by cell lysis with nonionic detergent in citric acid. Cell Struct Funct 1991;16: 203–207.
27 Green LC, Wagner DA, Glogowski J, Skipper PL, Wishnok JS, Tannenbaum SR: Analysis of nitrate, nitrite and [15N]nitrate in biological fluids. Anal Biochem 1982;126:131–138.
28 Chomczynski P, Sacchi N: Single-step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. Anal Biochem 1987;162:156–159.
29 Nunokawa Y, Ishida N, Tanaka S: Cloning of inducible nitric oxide synthase in rat vascular smooth muscle cells. Biochem Biophys Res Commun 1993;191:89–94.
30 Hart RP, Liu C, Shadiack AM, McCormack RJ, Jonakait GM: An mRNA homologous to interleukin-1 receptor type I is expressed in cultured rat sympathetic ganglia. J Neuroimmunol 1993;44:49–56.
31 Sabath DE, Broome HE, Prystowsky MB: Glyceraldehyde-3-phosphate dehydrogenase mRNA is a major interleukin 2-induced transcript in a cloned T-helper lymphocyte. Gene 1990;91:185–191.
32 Nishizawa M, Nakajima T, Yasuda K, Kanzaki H, Sasaguri Y, Watanabe K, Ito S: Close kinship of human 20alpha-hydroxysteroid dehydrogenase gene with three aldo-keto reductase genes. Genes Cells 2000;5:111–125.
33 Unezaki S, Nishizawa M, Okuda-Ashitaka E, Masu Y, Mukai M, Kobayashi S, Sawamoto K, Okano H, Ito S: Characterization of the isoforms of MOVO zinc finger protein, a mouse homologue of Drosophila Ovo, as transcription factors. Gene 2004;336:47–58.
34 Schreiber E, Matthias P, Müller MM, Schaffner W: Rapid detection of octamer binding proteins with mini-extracts, prepared from a small number of cells. Nucleic Acids Res 1989;17:6419.
35 Oda M, Sakitani K, Kaibori M, Inoue T, Kamiyama Y, Okumura T: Vicinal dithiol-binding agent, phenylarsine oxide, inhibits iNOS gene expression at a step of NF-κB DNA binding in hepatocytes. J Biol Chem 2000;275:4369–4373.
36 Bradford MM: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72:248–254.
37 Sakitani K, Kitade H, Inoue K, Kamiyama Y, Nishizawa M, Ito S, Okumura T: Anti-inflammatory drug sodium salicylate inhibits nitric oxide formation induced by interleukin-1β at a translational step, but not a transcriptional step, in hepatocytes. Hepatology 1997;25:416–420.
38 Inoue T, Kwon AH, Oda M, Kaibori M, Kamiyama Y, Nishizawa M, Ito S, Okumura T: Hypoxia and heat inhibit inducible nitric oxide synthase gene expression by different mechanisms in rat hepatocytes. Hepatology 2000;32:1037–1044.
39 Matsui K, Kawaguchi Y, Ozaki T, Tokuhara K, Tanaka H, Kaibori M, Matsui Y, Kamiyama Y, Wakame K, Miura T, Nishizawa M, Okumura T: Effect of active hexose correlated compound on the production of nitric oxide in hepatocytes. JPEN J Parenter Enteral Nutr 2007;31:373–381.
40 Pautz A, Linker K, Hubrich R, Korhonen R, Altenhofer S, Kleivert H: The polypyrimidine tract-binding protein (PTB) is involved in the post-transcriptional regulation of human inducible nitric oxide synthase expression. J Biol Chem 2006;281:32294–32302.
41 Ozaki T, Habara K, Matsui K, Kaibori M, Kwon AH, Ito S, Nishizawa M, Okumura T: Dexamethasone inhibits the induction of iNOS gene expression through destabilization of its mRNA in proinflammatory cytokine-stimulated hepatocytes. Shock 2010;33: 64–69.

Hepatology Research

Hepatology Research 2012; 42: 76–90 doi: 10.1111/j.1872-034X.2011.00891.x

Original Article

Japanese herbal medicine, inchinkoto, inhibits inducible nitric oxide synthase induction in interleukin-1β-stimulated hepatocytes

Takashi Matsuura,[1] Masaki Kaibori,[1] Yoshiro Araki,[1] Miho Matsumiya,[1] Yuka Yamamoto,[2] Yukinobu Ikeya,[3] Mikio Nishizawa,[4] Tadayoshi Okumura[1,5] and A-Hon Kwon[1]

[1]*Department of Surgery, Kansai Medical University, Moriguchi, Osaka, and* [2]*Graduate School of Science and Engineering, and* [3]*Departments of Pharmacy, College of Pharmaceutical Sciences and* [4]*Biomedical Sciences, College of Life Sciences, and* [5]*Research Organization of Science and Technology, Ritsumeikan University, Kusatsu, Shiga, Japan*

Aim: A herbal medicine, kampo inchinkoto (TJ-135), is used to treat jaundice and liver fibrosis in patients with cirrhosis. In the inflamed liver, proinflammatory cytokines stimulate the induction of inducible nitric oxide synthase (iNOS) gene expression. Over-production of nitric oxide (NO) by iNOS has been implicated as a factor in liver injury. We examined interleukin (IL)-1β-stimulated hepatocytes as a simple *in vitro* injury model to determine liver-protective effects of TJ-135. The objective was to investigate whether TJ-135 influences iNOS induction and to determine its mechanism.

Methods: Primary cultured rat hepatocytes were treated with IL-1β in the presence or absence of TJ-135. The induction of iNOS and its signaling pathway were analyzed.

Results: IL-1β produced increased levels of NO. This effect was inhibited by TJ-135, which exerted its maximal effects at 3 mg/mL. TJ-135 decreased the levels of iNOS protein and its mRNA expression. Experiments with nuclear extracts revealed that TJ-135 inhibited the translocation of nuclear factor-κB (NF-κB) to the nucleus and its DNA binding. TJ-135 also inhibited the activation of Akt, resulting in the reduction of type I IL-1 receptor mRNA and protein expression. Transfection experiments with iNOS promoter-luciferase constructs demonstrated that TJ-135 suppressed iNOS induction by inhibition of promoter transactivation and mRNA stabilization. TJ-135 reduced the expression of an iNOS gene antisense-transcript. Delayed administration or withdrawal of TJ-135 after IL-1β addition also inhibited iNOS induction.

Conclusions: Results indicate that TJ-135 inhibits the induction of iNOS at both transcriptional and post-transcriptional steps, leading to the prevention of NO production. TJ-135 may have therapeutic potential for various liver injuries through the suppression of iNOS induction.

Key words: inducible nitric oxide synthase, interleukin-1β, liver injury, nuclear factor-κB, primary cultured hepatocytes, type I interleukin-1 receptor

INTRODUCTION

JAPANESE TRADITIONAL HERBAL medicines (Kampo) have been empirically administered by clinicians to patients with a variety of diseases. One such medicine, inchinkoto (TJ-135), is traditionally used for icteric patients with cirrhosis, and also used as an anti-inflammatory, antipyretic, choletic and diuretic agent for liver disorders and jaundice. TJ-135 is an aqueous extract from three herbs: *Artemisia capillaris spica, Gardenia fructus* and *Rhei rhizome* with a weight ratio of 4:3:1, which is now manufactured under modern scientific quality controls. *A. capillaris* and *G. fructus* are effective for liver diseases, and *R. rhizome* is a laxative. It has been reported that TJ-135 was used to improve acute hepatitis of unknown etiology, but the mechanism is unknown.[1,2] TJ-135 is considered as a choleretic and hepatoprotective agent with relevant effects on bile formation,[3] hepatic oxidative stress, hepatic fibrogenesis and stellate cell apoptosis.[4–6]

However, there is little scientific evidence to demonstrate the liver-protective effects of TJ-135. In hepatic disorders, inflammatory cells such as platelets and macrophages gather around hepatic stellate cells and

Correspondence: Dr Tadayoshi Okumura, Department of Surgery, Kansai Medical University, 10-15 Fumizonocho, Moriguchi, Osaka 570-8506, Japan. Email: okumura@takii.kmu.ac.jp
Received 11 May 2011; revision 10 August 2011; accepted 16 August 2011.

discharge cytokines. During inflammation, pro-inflammatory cytokines and nitric oxide (NO) produced by inducible nitric oxide synthase (iNOS) play an important role as factors in liver injury.[7] However, definition of the role of NO is confounded by reports that it can exert either detrimental or beneficial effects depending on the insults and tissues involved.

We have previously reported that in animal liver injury models caused by various insults, such as ischemia-reperfusion, partial hepatectomy and endotoxin shock, the induction of iNOS and NO production is upregulated concomitantly with the production of pro-inflammatory cytokines in the liver.[8–12] In these studies, drugs showing liver-protective effects inhibited the induction of iNOS and NO production as well as the decreased production of various inflammatory mediators. Furthermore, *in vitro* experiments with primary cultured rat hepatocytes revealed that these drugs also inhibited the induction of iNOS and the production of NO.[10,13,14] Thus, downregulating NO production is considered to be an indicator of liver protection. In this study, we used interleukin (IL)-1β-stimulated cultured hepatocytes as a simple *in vitro* injury model to investigate the liver-protective effects of TJ-135 for *in vivo* animal models. We investigated whether TJ-135 directly influences iNOS induction in cultured hepatocytes and the mechanism involved.

MATERIALS AND METHODS

Materials

INCHINKOTO (TJ-135) was provided by Tsumura Co., Ltd. (Tokyo, Japan). TJ-135 was dissolved in Williams' medium E (WE) and vortexed for 30 min at room temperature, followed by centrifugation (11 000 *g* for 15 min). The supernatant was filter-sterilized with a 0.45-μm membrane filter (Millipore, Billerica, MA, USA) prior to use in experiments. TJ-135 components (*A. capillaris*, *G. fructus* and *R. rhizome*) were purchased from Tochimoto Tenkaido Co., Ltd. (Osaka, Japan), and were extracted with water under reflux for one hour. Extracted solutions were freeze-dried to obtain the water extracts; 0.85 g, 2.67 g and 1.61 g from *A. capillaris*, *G. fructus* and *R. rhizome* (each 10 g), respectively.

Recombinant human IL-1β (2×10^7 U/mg protein) was provided by Otsuka Pharmaceutical Co., Ltd. (Tokushima, Japan). [γ-^{32}P]-Adenosine-5′-triphosphate (ATP; 222 TBq/mmol) was obtained from DuPont-New England Nuclear Japan (Tokyo, Japan). Rats were kept at 22°C under a 12:12 h light : dark (LD) cycle, and received food and water *ad libitum*. All animal experiments were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals of the National Institutes of Health, and approved by the Animal Care Committee of Kansai Medical University.

Primary cultures of hepatocytes

Hepatocytes were isolated from male Wistar rats (200–220 g; Charles River, Tokyo, Japan) by perfusion with collagenase (Wako Pure Chemicals, Osaka, Japan).[15,16] Isolated hepatocytes were suspended in culture medium at 6×10^5 cells/mL, seeded into 35-mm plastic dishes (2 mL/dish; Falcon Plastic, Oxnard, CA, USA) and cultured at 37°C in a CO_2 incubator under a humidified atmosphere of 5% CO_2 in air. The culture medium was WE supplemented with 10% newborn calf serum, Hepes (5 mM), penicillin (100 U/mL), streptomycin (0.1 mg/mL), dexamethasone (10 nM) and insulin (10 nM). After 5 h, the medium was replaced with fresh serum- and hormone-free WE, and the cells were cultured overnight before use in experiments. The numbers of cells attached to the dishes were calculated by counting the number of nuclei[17] and using a ratio of 1.37 ± 0.04 nuclei/cell (mean ± SE, $n = 7$ experiments).

Treatment of cells with TJ-135 and its components

On day 1, the cells were washed with fresh serum- and hormone-free WE, and incubated with IL-1β (1 nM) in the same medium in the presence or absence of TJ-135 and its components. The doses of TJ-135 and its components used are indicated in the appropriate figures and their legends.

Determination of NO production and lactate dehydrogenase activity

Culture medium was used for measurements of nitrite (a stable metabolite of NO) to reflect NO production by the Griess method.[18] Culture medium was also used for measurements of lactate dehydrogenase (LDH) activity to reflect cell viability using a commercial kit (Wako Pure Chemicals).

Western blot analysis

Total cell lysates were obtained from cultured cells as described previously[13] with minor modifications. Briefly, cells (1×10^6 cells/35-mm dish) were lysed in 100–200 μL of solubilizing buffer (10 mM Tris-HCl, pH 7.4, containing 1% Triton X-100, 0.5% Nonidet P-40, 1 mM ethylenediaminetetraacetic acid [EDTA], 1 mM ethyleneglycol bis (2-aminoethyl ether) tetraacetic acid (EGTA), phosphatase inhibitor cocktail [Nacalai